

*A Jigsaw Puzzle: Detecting Structural Changes in ykkCD Riboswitch RNA in  
Response to Tetracycline Binding*

**An Honors Thesis (HONR 499)**

**by**

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## Abstract

Each year in the United States, at least 2 million people become infected with bacteria that are resistant to antibiotics. Approximately 23,000 people die each year as a direct result of these infections, which makes antibiotic resistance an important topic of research. The Gerczei lab is investigating a mechanism of antibiotic resistance that involves the ykkCD riboswitch. This riboswitch was first discovered in *Bacillus subtilis*, and it specifically recognizes the tetracycline family of antibiotics. When tetracycline is present in the cell, the antibiotic induces synthesis of the riboswitch mRNA which leads to the production of an efflux pump. This pump serves as a transporter protein and pumps the antibiotic out of the cell, rendering the cell resistant to tetracycline. The objective of this project is to detect structural changes occurring within the secondary structure of the ykkCD riboswitch RNA in response to tetracycline binding using nucleic acid footprinting methods. Ultimately, the hope is to use these footprinting techniques to determine the binding site of tetracycline on the RNA and map the structural change caused by tetracycline binding.

## **Acknowledgements**

First of all, I would like to thank my parents for always showing me their fullest support and encouraging me in all of my personal and academic endeavors. I truly would not have been able to reach the point that I am at today without you.

Secondly, I would like to thank Dr. Timea Gerczei for allowing me to join her laboratory and guiding me through this research process. Your direction and continued support helped make this project possible.

I would also like to thank Dr. Paul Coan for providing students like myself with the opportunity to participate in summer research through the CRISP Program.

Lastly, thank you to Mark Buckles and all of the other students in the lab who helped make all of the hours in the lab an enjoyable experience.

## **Process Analysis Statement**

I completed an independent research project under the advisory of Dr. Timea Gerczei to study how the binding of the antibiotic tetracycline affects the secondary structure of the ykkCD RNA. Antibiotic resistance is an ever-growing problem, and tetracycline is known to induce a resistance mechanism within bacterial cells that contain ykkCD RNA. When tetracycline enters the cell it binds to the RNA, which induces a structural change in the RNA that allows transcription to continue. As a result, a multi-drug resistance efflux pump is produced that pumps the antibiotic out of the cell. Therefore, the structural change that occurs within the RNA to allow for pump production is of interest since it results in resistant bacterial cells.

This research project was both challenging and exciting for me. I was able to utilize many of the laboratory techniques that I previously learned in the classroom, and I was exposed to new techniques that are commonly used in professional biochemistry laboratories. I enjoyed being able to directly apply my education in an independent setting. The project was not without challenges. As with much scientific research, it took a large amount of time and repetition to collect results. It was difficult to not be discouraged when long hours of meticulous work would produce little to no data. However, I was able to improve as a researcher and appreciate small victories.

The project served as a valuable extension of my education, and it will also be useful for future researchers in the chemistry department at Ball State University. I hope that future students will be able to use this data to further research the secondary structure of ykkCD RNA. Studies regarding antibiotic resistance will continue to be an important area of research in the future, and I am proud to have participated in a small part of it.



## Introduction

### *Discovery of Antibiotics*

The age of antibiotics began in 1928 when Alexander Fleming, Professor of Bacteriology at St. Mary's Hospital in London, discovered penicillin. Antibiotics are produced by fungi or bacteria and have the ability to inhibit or kill other microbial species. Fleming was studying colonies of *Staphylococcus aureus* in petri dishes when he noticed that there was an area of inhibited growth surrounding a mold. This mold, later identified as *Penicillium notatum*, secreted a substance that was capable of killing different harmful bacteria, including *streptococcus* and *meningococcus*.<sup>1</sup>

Following Fleming's work, more research was conducted at Oxford University to further the fermentation and purification techniques used for penicillin production. In 1941, Albert Alexander was the first patient to receive penicillin. Research then moved to the United States in 1941 and several pharmaceutical companies, including Lilly and Pfizer, began to mass-produce penicillin after developing more efficient growth and fermentation methods.<sup>1</sup> Penicillin quickly became a key component of the war effort during that time, used to treat many soldiers with bacterial infections during World War II. Therefore, large-scale production of penicillin incited the age of antibiotics and a revolution within the pharmaceutical industry. Other antibiotics began to be discovered and developed, including tetracycline, erythromycin, and methicillin. However, at the very beginning of the antibiotic age, Fleming warned that "the public will demand [the drug and]...then will begin an era...of abuses," which he proposed would lead to eventual bacterial resistance.<sup>15</sup>

### *History of Antibiotic Resistance*

Just as Fleming cautioned years earlier, bacteria adapted and became resistant to penicillin by the 1950s. Since then many other antibiotics have been discovered, only to later be counteracted by antibiotic-specific bacterial resistance. Unfortunately, resistant strains of bacteria have been identified for the majority of antibiotics that have been developed since the 1940s. There are several potential causes behind the emergence of resistant strains of bacteria. One of the main causes is simply overuse. Studies have shown that there is a direct correlation between consumption of antibiotics and the development of bacterial resistance. Because antibiotics are produced by bacteria themselves, these bacteria are inherently capable of mutating and adapting in order to survive. When antibiotics are consumed any drug-sensitive bacteria are killed and resistant bacteria are left behind to grow and reproduce. Therefore, overuse of antibiotics leads to increased presence of resistant bacteria.<sup>15</sup>

Another cause of overuse is inappropriate prescribing by primary physicians and hospitals. Studies done previously indicate that treatment indication, choice of antibiotic, or duration of therapy is wrong in 30-50% of cases. Another study suggested that 30-60% of antibiotics prescribed in intensive care units are unnecessary or inappropriate. When antibiotics are prescribed incorrectly, they are more detrimental than beneficial and can promote resistance by inducing mutagenesis and altered gene expression. Additionally, resistant bacteria can be passed to humans through food consumption, specifically meat products. Antibiotics are commonly given to livestock as growth supplements to prevent infection and promote growth. While this method can lead to higher quality products, it also contributes to the growing antibiotic resistance crisis. When livestock are given

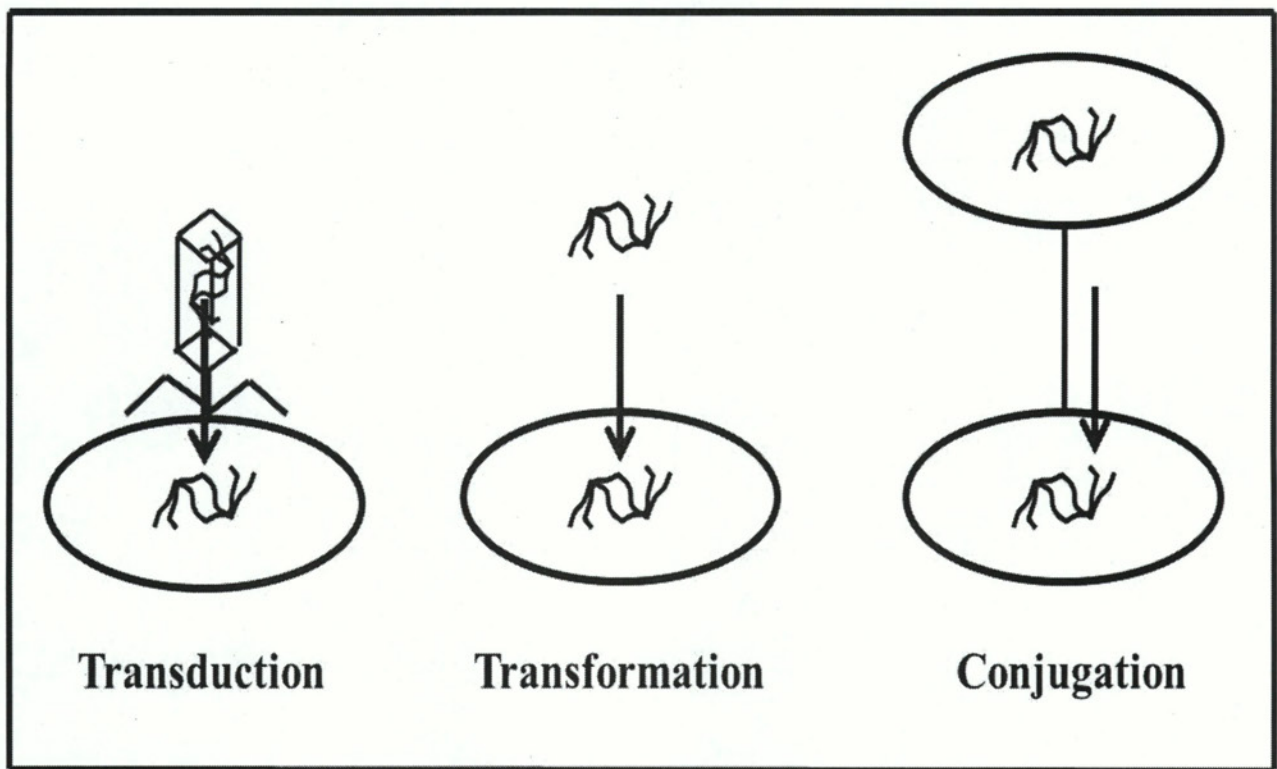


antibiotics only the resistant bacteria survive and they can then be transmitted to the humans who consume those food products.<sup>15</sup>

Lastly, the lack of availability of new antibiotics plays a role in the current crisis. When antibiotics were first discovered researchers were able to discover and develop new drugs when resistance to a previous antibiotic would be discovered. Now, however, new antibiotics are not being identified as readily due to economic and regulatory obstacles. For several pharmaceutical companies antibiotic research is not a solid investment since these drugs are used for such a short period of time and are often remedial, which makes them not as profitable. Similarly, different regulatory changes made by the FDA over the past few decades have made clinical studies much more expensive for pharmaceutical companies. Therefore, fewer companies are conducting research and fewer antibiotics are being discovered, which makes it more difficult to counter the ongoing battle against resistant bacteria.<sup>15</sup>

#### *Mechanisms of Antibiotic Resistance*

In order to fight against antibiotics, bacteria employ two main methods to adapt to their respective antibiotic: mutations in genes or attainment of external genetic material that is already resistant. Bacteria can obtain resistant foreign DNA by three different methods: transformation, conjugation, and transduction (Figure 1). Transformation involves the incorporation of free exogenous DNA into a recipient cell. Conjugation is the transfer of genetic material via cell-to-cell contact. Plasmids containing the resistant gene are passed from one cell into the recipient cell. During transduction, the resistant gene is introduced into the cell via viral delivery.<sup>2</sup>

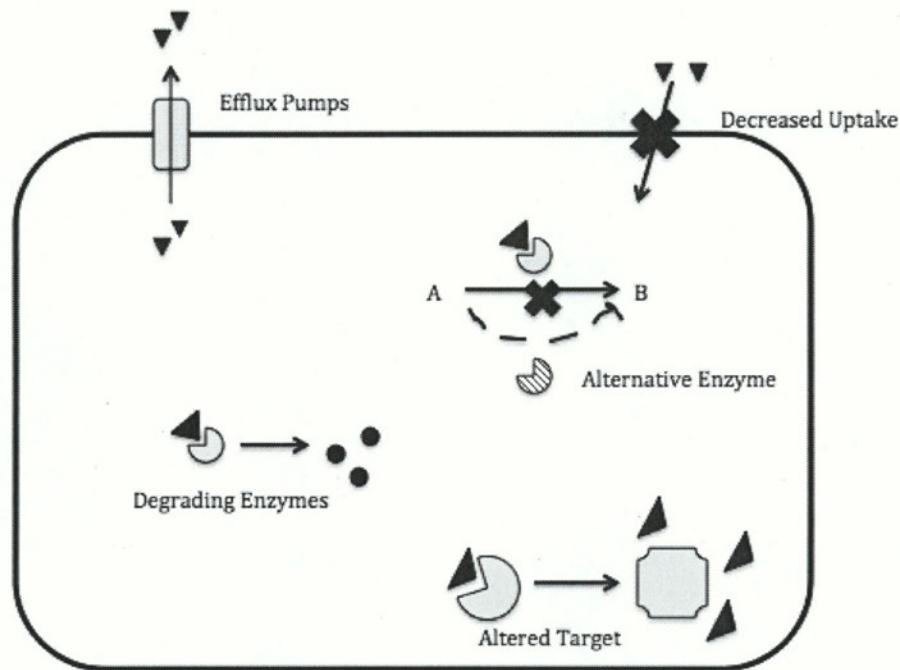


**Figure 1.** Schematic of the three methods involved in horizontal gene transfer of resistant genes into bacteria.<sup>13</sup>

Once a bacterial cell becomes resistant due to a mutation or one of the above methods of gene transfer, there are several mechanisms that are employed to counteract the presence of antibiotics (Figure 2). The first resistance mechanism is the chemical alteration or destruction of the antibiotic molecule. In this case, the bacteria cell produces enzymes that are able to alter the molecule through acetylation, phosphorylation, and adenylation. The addition of these different chemical moieties results in a decreased level of avidity for the antibiotic and its intended target. Similarly, the cell can also produce enzymes to break down the antibiotic so that it is never able to reach its target. The second resistance mechanism involves the decreased permeability of the cell membrane and therefore decreased uptake of the antibiotic molecules. The third resistance mechanism works by protecting the target site by bypassing it altogether. The cell is able to produce an



alternative enzyme that can be used instead of the protein that is inhibited by the antibiotic. Therefore, the antibiotic binds to the alternate protein and isn't able to bind to its intended protein so the cell survives. Similarly, the cell can induce modifications of the target site, which results in decreased affinity of the antibiotic for its target. Lastly, a cell can resist the effects of antibiotics by pumping the antibiotic out of the bacterial cell through transporter proteins known as efflux pumps.<sup>10</sup>



**Figure 2.** Model of the antibiotic resistance mechanisms mentioned above.<sup>12</sup>

### *Efflux Pumps*

An efflux pump system that extruded tetracycline from bacterial cells was one of the first to be described in the early 1980s.<sup>10</sup> Efflux pumps are transport proteins that expel toxic substances from inside the cell to the external environment. In this case, bacterial cells are able to pump out antibiotics that threaten the survival and growth of the cell. These pumps can be specific to one antibiotic or they can transport a range of different compounds, which contributes to the threat of multiple drug resistance. There are five

major families of these transporter proteins, and the majority of them use proton motive force as the energy source behind the expulsion of toxic substances.<sup>4</sup>

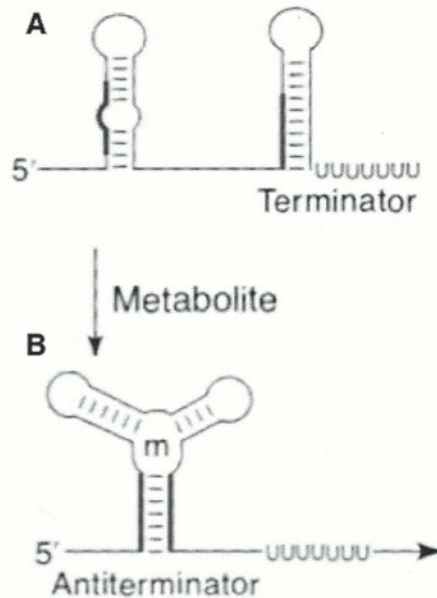
These efflux pump proteins have been found to be encoded in both antibiotic susceptible and resistant bacteria. In non-resistant bacteria, efflux pumps are often part of an operon and have tightly regulated gene expression. However, in resistant bacteria overexpression of efflux pumps is associated with resistance. Genes encoding these proteins can be found on chromosomes or plasmids; however, when located on the chromosome the bacterium has an intrinsic mechanism to fight against antibiotics instead of requiring horizontal gene transfer.<sup>16</sup> This intrinsic mechanism becomes activated when the bacterium detects the antibiotic molecule in its environment via ligand binding by transcription factors or riboswitches.

#### *Riboswitches*

A riboswitch is defined as a metabolite-binding domain within the messenger RNA that influences gene expression in response to ligand binding. These regulatory domains are highly conserved across prokaryotes and are found within the 5' untranslated region of the bacterial mRNA transcript.<sup>17</sup> A riboswitch is composed of two domains: the aptamer domain and the expression platform. The specific metabolite binds to the aptamer domain when the metabolite concentration reaches its threshold, which induces a conformational change within the expression platform. This structural change leads to modified expression of genes carried by the mRNA.<sup>17</sup>

In the majority of cases, metabolite binding to the riboswitch results in decreased expression of the gene due to transcription termination or translation inhibition. When the metabolite binds, the structural change results in the formation of a terminator hairpin that

destabilizes the complexes needed for elongation, which leads to attenuation. However, some riboswitches upregulate gene expression in response to metabolite binding.<sup>17</sup> In this case, the conformational change seen in Figure 3 occurs within the riboswitch mRNA.



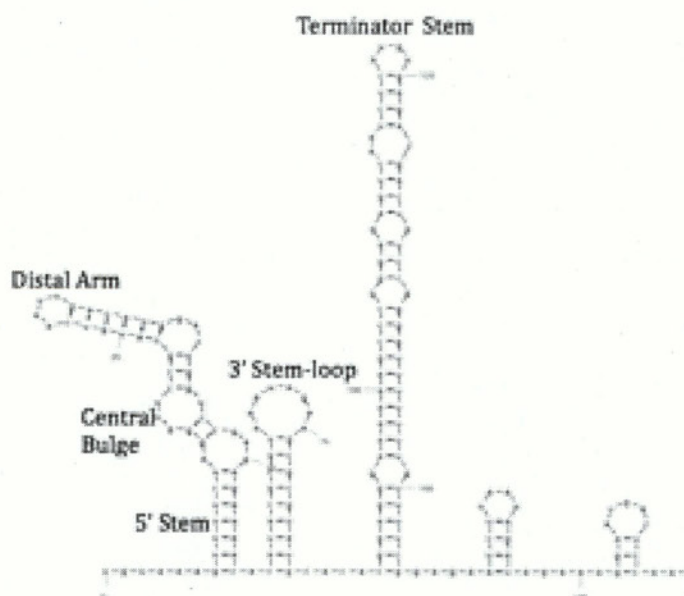
**Figure 3.** Schematic representation of upregulation of gene expression in response to metabolite binding to riboswitch mRNA.

As seen in Figure 3A, when the metabolite is not present in the cellular environment, the terminator stem is intact. The mRNA is released at that point and there is no expression of the gene directly downstream. In Figure 3B, the metabolite is now present and binds to the aptamer domain of the riboswitch, which leads to a conformational change that unfolds the terminator stem and produces an antiterminator. Transcription is allowed to continue and expression of the downstream gene is upregulated.<sup>3</sup>

There are currently 12 different classes of riboswitches discovered, but this research project focuses specifically on the ykkCD toxin sensor RNA, which is a riboswitch that recognizes tetracycline. This riboswitch was first discovered in *Bacillus subtilis*. The



ykkCD riboswitch is a highly conserved portion of mRNA that regulates the expression of the ykkCD multidrug resistant efflux pump (MDR pump). As explained above, an MDR pump is able to expel a variety of toxic substances out of a cell, more specifically antibiotics that threaten cell survival. When the pump is expressed in a bacterium, the antibiotic is removed from the cell thus rendered ineffective. The ykkCD riboswitch mRNA is found upstream of the gene that encodes for the ykkCD MDR efflux pump. As seen in Figure 4, when tetracycline is not present, the ykkCD riboswitch remains in the “off” position with the terminator stem intact so the gene encoding the pump is not expressed. When tetracycline is introduced into the cell and reaches its threshold, it can bind to the aptamer domain of the riboswitch, which is found 5' to the expression platform. This leads to the unfolding of the terminator stem, which allows expression of the downstream gene. The pump protein is then produced and can actively expel tetracycline from the cell.<sup>6</sup>



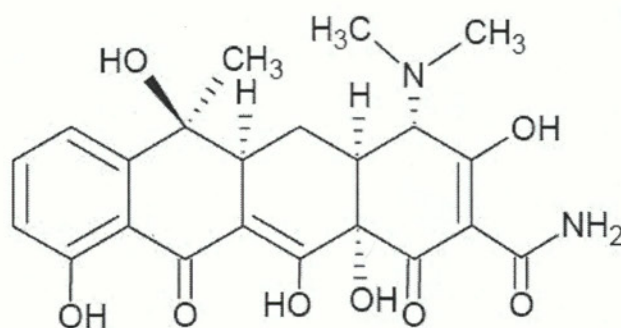
**Figure 4.** Proposed secondary structure of the ykkCD riboswitch aptamer predicted by *mfold*, a computational molecular biology program.



As seen in Figure 4, the ykkCD riboswitch contains the anticipated aptamer domain on the left and the expression platform on the right.

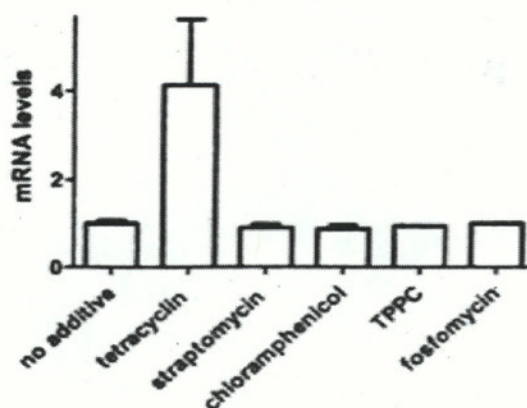
#### *Tetracycline Family Antibiotics*

As stated previously, the specific target of the ykkCD riboswitch is tetracycline (Figure 5). This antibiotic is used to treat bacterial infections, such as pneumonia and other respiratory infections as well as acne and other skin infections.<sup>14</sup> Tetracyclines were discovered in the 1940s and fight bacteria by inhibiting protein synthesis. These antibiotics are relatively inexpensive, which has led to a high rate of prescription and therefore resistance.<sup>5</sup>



**Figure 5.** Chemical structure of tetracycline.

In order to determine whether the ykkCD riboswitch specifically binds tetracycline to induce production of the efflux pump, previous research was done to analyze ykkCD efflux pump levels in the presence of a variety of ligands. Tetracycline, streptomycin, chloramphenicol, TPPC, and fosfomycin are ligands of the ykkCD efflux pump; therefore, all of these ligands were tested to see whether they triggered expression of the pump. When *B. subtilis* cells were grown in the presence of each of these substances, the following information in Figure 6 was collected.<sup>6</sup>



**Figure 6.** Figure of mRNA levels of the *ykkCD* pump in response to a variety of antibiotics. Adapted from Caronline Conley's research.<sup>6</sup>

As seen in Figure 6, the level of *ykkCD* pump mRNA was highest in the presence of tetracycline, which supports the hypothesis that tetracycline specifically binds to induce expression of the *ykkCD* efflux pump.

Similarly, previous research was done by Delores James using binding assays with four derivatives of tetracycline to determine which tetracycline functional groups are essential for recognition. The four derivatives studied were minocycline, anhydrotetracycline, oxytetracycline, and doxycycline. Tetracycline and these derivatives are naturally fluorescent, so once the antibiotic binds to the sensor it undergoes quenching and the fluorescence decreases. According to these binding assays, it was found that minocycline and anhydrotetracycline bind to the riboswitch in addition to tetracycline.<sup>8</sup> However, oxytetracycline and doxycycline did not bind. As seen in Figure 7, both minocycline and anhydrotetracycline alter the lower face of tetracycline, which suggests that the lower side is not as important to recognition since these two derivatives both still bind the riboswitch. On the other hand, both oxytetracycline and doxycycline alter the upper side of the antibiotic, which suggests that this upper area of tetracycline affects the

ability for recognition since these two derivatives did not bind.<sup>8</sup> In order to continue studying the binding of tetracycline to the ykkCD riboswitch mRNA, several probing techniques are used to analyze the conformational changes that occur in response to ligand binding.



**Figure 7.** Chemical structures of tetracycline and its derivatives used in a binding assay to study direct and specific binding of tetracycline to the ykkCD riboswitch.

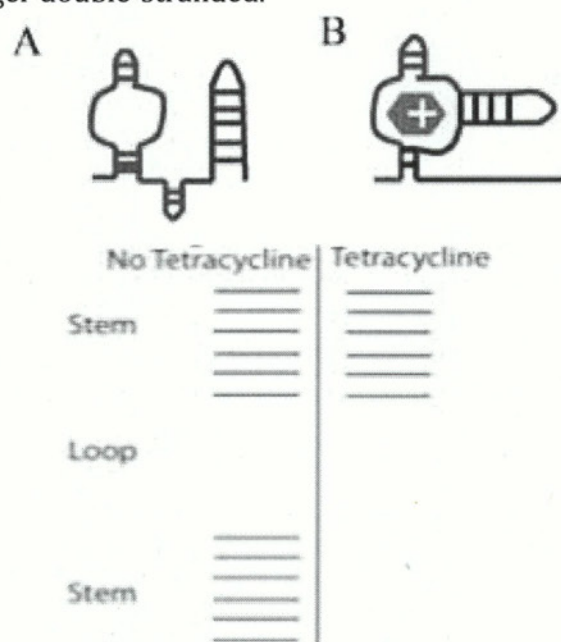
#### Structure Probing Techniques

RNA footprinting is an enzymatic method of mapping RNA structure to monitor ligand binding and conformational changes.<sup>7</sup> By subjecting the RNA of interest to ribonuclease digestion, the subsequent cleavage pattern can be determined and analyzed using a thin gel called a sequencing gel. The distinct cleavage bands that appear on the sequencing gel make up the “footprint.” There are several probing techniques that can be used for RNA footprinting, but the one studied here is ribonuclease protection.

A variety of different enzymes with different substrate specificities are used to study the digestion products with and without ligand bound. A comparison of the ykkCD RNA and the ykkCD RNA-tetracycline complex footprints will reveal where tetracycline binds to the



ykkCD RNA. The region of RNA where tetracycline binds will be protected from digestion by the bound ligand, resulting in the absence of distinct bands in those areas on the sequencing gel. In order to visualize these cleavage patterns, the RNA is subjected to reverse transcription using a labeled primer. The resulting end-labeled DNAs are resolved on a sequencing gel. An example of a sequencing gel that detects structural changes in the presence of RNase V1 can be seen in Figure 8. RNase V1 specifically cleaves double-stranded RNA. When there is no tetracycline present, the terminator stem is intact and the enzyme cleaves the double-stranded RNA, which is illustrated on the left hand side of Figure 8. When tetracycline is present, the stem unfolds and the cleavage bands disappear since the DNA is no longer double-stranded.

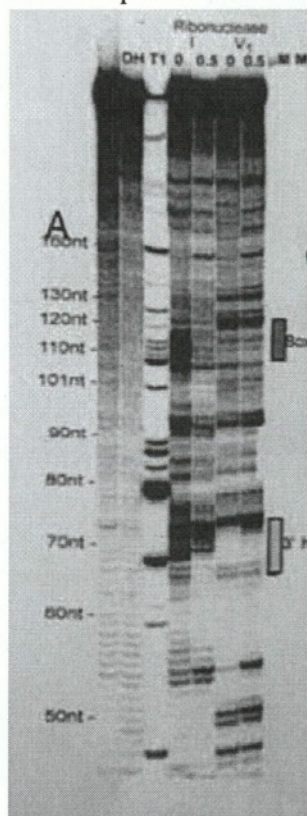


**Figure 8.** Schematic of DNA illustrating the structural change that takes place when the antibiotic is introduced into the environment. These changes can be detected on a sequencing gel by analyzing the changes in the cleavage patterns in conjunction with the knowledge of the cleaving specificity of each RNase enzyme.

Ribonuclease protection assays are a sensitive and specific method used for the detection, mapping, and quantitation of specific nucleotides in an RNA sequence.<sup>11</sup> There



are a variety of ribonucleases available, but those used in this research project are RNase T1, V1, and 1. Each of these cleave RNA after specific nucleotides and at specific sites. RNase T1 cleaves at the 3' end of single-stranded G nucleotides. As mentioned previously, RNase V1 cleaves base paired nucleotides. RNase 1 cleaves all RNA dinucleotide bonds, which produces a 5' hydroxyl and 2',3' cyclic monophosphate. In conjunction with these RNases, an OH ladder and a T1 ladder are also essential in order to effectively analyze the cleavage pattern on a denaturing gel. The hydroxyl (OH) ladder cleaves after every nucleotide while the T1 ladder cleaves after every G nucleotide. The combination of these two ladders provide a method of identifying which nucleotides are being protected or altered due to ligand binding. A sample sequencing gel image can be seen in Figure 9, which shows the cleavage pattern of a sample of a ribonuclease protection assay.



**Figure 9.** Sample sequencing gel done by Dr. Timea Gerczei that shows the cleavage patterns of ribonuclease 1 and V1 versus an OH ladder and T1 ladder.

### *Determining Tetracycline Binding Site Using RNase Protection*

I conducted an independent research project to investigate and analyze the tetracycline binding site on the ykkCD riboswitch. The purpose of the study is to subject the ykkCD RNA to ribonuclease protection assays both with and without tetracycline in order to determine how ligand binding affects the cleavage pattern of the RNA. I chose to focus on RNase protection assays instead of other probing techniques because they are very sensitive to secondary structural changes than other techniques. These nucleases have specificity in their ability to cleave so the cleavage patterns should change in a synchronized manner if structural change occurs and they should change in the same way when tetracycline binding occurs. These are significant benefits because it is important to be able to determine how ligand binding affects the secondary structure of the RNA in order to determine how tetracycline binding leads to alteration of gene expression.

The RNase protection assays are also relatively easy to optimize because they have a set pH and buffer conditions. Therefore, it only requires optimization of the amount of nuclease that is required to produce the most indicative cleavage pattern. The sites where tetracycline binds to the RNA will be protected from nuclease digestion, which results in the absence of a band or a less intense band on a denaturing gel. In this research project, the cleavage patterns will be visualized using reverse transcription with labeled primers. Another option would be to use radiolabeling with nucleotides containing  $^{32}\text{P}$ . This is a common technique used for nucleic acid labeling, but the radiolabeled nucleotides are expensive and decay quickly. Therefore, it was not conducive to use this technique in an undergraduate lab where the radiolabeled RNA would not be able to be used immediately following synthesis. Therefore, the cleavage patterns will be visualized using reverse

transcription since it is more cost effective and conducive to the hours spent in the undergraduate lab. By analyzing these cleavage pattern on a sequencing gel in conjunction with an OH ladder and T1 ladder, the nucleotides that are affected by tetracycline binding can be identified.

## **Materials and Methods**

### *ykkCD Riboswitch RNA Synthesis*

To design the ykkCD RNA of interest, the DNA template was first amplified using PCR technology. The DNA template for the ykkCD RNA is as follows:

TAATACGACTCACTATAGGCCTTCGGGCCAATGTAAAGTTTCTAGGGTTCCGCATGTCAATTGA  
CATGGACTGGTCCGAGAGAAAAACACATACGCGTAAATAGAAGCGCGTATGCACACGGAGGGAAA  
AAAGCCCGGGAGAGTCGATCCGGTTCGCCGGATCCAAATCGGGCTTCGGTCCGGTTC

where the underlined portion of the template is the T7 polymerase promoter sequence.

Following the instructions of the manufacturer, the following components were added in a 1.5mL centrifuge tube to make the PCR master mix: 1  $\mu$ L DNA template, 10  $\mu$ L 10 mM top primer, 10  $\mu$ L 10 mM bottom primer, 200  $\mu$ L 5x reaction buffer, 20  $\mu$ L 10mM dNTP mix, 739  $\mu$ L water, and 20  $\mu$ L Thermo Scientific PHIRE Hot Start II DNA Polymerase. This solution was then aliquoted into 10 0.5mL tubes, which were placed in the Eppendorf Authorized Thermal Cycler to undergo PCR amplification. The series of steps involved in PCR amplification are outlined in Table 1.



**Table 1.** Outline of PCR reaction steps using the PHIRE program on the Eppendorf Authorized Thermal Cycler.

	Temperature (°C)	Time (sec)	Action
Step 1	98	30	Begin amplification process
Step 2	98	5	Separate the DNA strands
Step 3	39	5	Anneal primers
Step 4	72	15	Amplification
Repeat steps 2-4 for a total of 35 times – yields a large amount of DNA			
Step 5	72	60	Final amplification
Step 6	4	infinite	Allows storing

Once the amplification was complete, the PCR product was purified using the Qiagen PCR Cleanup Kit per suggestion of the manufacturer. The PCR products were transferred into a 10mL tube and 5mL of PB buffer was added and mixed. Using two PCR cleanup columns, 700  $\mu$ L of product was transferred into each column and centrifuged for two minutes at low speed. The materials that flowed through the column were discarded. This step was repeated several times until all of the PCR product had been added to the cleanup column. Each column was then washed with 700  $\mu$ L PE buffer, centrifuged as before, and the flow through material was discarded. The columns were centrifuged for 1 minute at maximum speed, which removes ethanol from the PE buffer. The subsequent flow through material was discarded. The DNA was then eluted from the column. The column was placed in a clean centrifuge tube and 50  $\mu$ L of elution buffer were pipetted onto the middle of the column. After a wait period of 1 minute, the tube was centrifuged for 1 minute at maximum speed. The DNA concentration was determined using the Thermo Scientific Nanodrop 1000 Spectrometer with a typical yield of approximately 200 ng/ $\mu$ L.

The DNA quality was checked using agarose gel electrophoresis. From a 40x stock, a 1x TAE buffer was made and 100  $\mu$ L was added to an Erlenmeyer flask along with 1.0g



agarose. The solutions was microwaved for 2 minutes until all agarose was dissolved. Ethidium bromide (10  $\mu$ L, 10mg/mL) was added to the flask and the gel solution was poured into the casting gel. Upon solidification, 700  $\mu$ L of 1x TAE buffer was added and two samples were loaded side by side: 5  $\mu$ L of a 50 bp molecular weight marker with 2  $\mu$ L loading dye and 5  $\mu$ L of the DNA sample with 2  $\mu$ L loading dye. The gel was run for 20 minutes at 100V. The gel was imaged using the Biorad Molecular Imager on the ethidium bromide setting. A solitary strong band around 200 bp is indicative of a high quality DNA sample.

Following the production of ykkCD DNA, transcription was completed to yield the ykkCD RNA of interest. A 500  $\mu$ L transcription mix was made using the following components:

- 50  $\mu$ L 10X reaction buffer
- 10  $\mu$ L rNTP mix
- 52  $\mu$ L DNA
- 25  $\mu$ L DTT
- 50  $\mu$ L T7 RNA Polymerase
- 313  $\mu$ L MilliQ water

This reaction mix was placed in a 37°C water bath for 2 hours for transcription to occur. The DNA template was then removed using 10  $\mu$ L of RNase free DNase and allowed to incubate for another 30 minutes. The remaining white pellet was dissolved using 50  $\mu$ L 0.5 M EDTA (pH ~ 8.0).

The transcribed RNA was precipitated using phenol/chloroform extraction and ethanol precipitation in order to reduce RNA volume and remove buffer, enzyme and unincorporated nucleotides. For the extraction, 500  $\mu$ L phenol/chloroform mix was added to the RNA to remove the polymerase. After vortexing and centrifugation, the top layer was

transferred into a centrifuge tube and 500  $\mu$ L of chloroform was added to remove the phenol. After vortexing and centrifugation, the top layer was transferred to a new tube and underwent ethanol precipitation, which includes the following additions to the tube:

- 50  $\mu$ L 3M NaOAc (pH ~ 3.5)
- 500  $\mu$ L isopropanol
- 1  $\mu$ L glycogen (20 mg/mL)

The solution was incubated overnight at -20°C. Following the incubation period, the RNA was recovered from the solution using the following protocol:

- Centrifuge sample at maximum speed at 4°C for 30 minutes
- Remove supernatant and discard – RNA is at the bottom of the tube
- Add 100  $\mu$ L ice-cold 75% ethanol, centrifuge for 5 minutes at 4°C and discard supernatant
- Dry pellet in Labconco speed-vac for 5 minutes
- Dissolve RNA in 100  $\mu$ L RNase-free water

The recovered RNA was purified using a denaturing urea PAGE gel in order to remove any contamination from the RNA including lower molecular weight degradation products. The components used for the gel solution, urea dye, and running buffer can be seen in Table 2.

**Table 2.** Volumes and contents of the gel solution, urea dye, and running buffer used for gel purification of the recovered RNA.

Gel Solution	0.5X Urea Dye	0.5X TBE Running Buffer
3.6 g urea	0.36 g urea	50 mL 10X TBE buffer
0.5 mL 10x TBE buffer	100 $\mu$ L 5X TBE buffer	Water to 1 L mark
5 mL 40% acrylamide/bisacrylamide	10 $\mu$ L bromophenol blue	
100 $\mu$ L 10% ammonium persulfate	Water to 1 mL mark	
10 $\mu$ L TEMED		

Once the gel was prepared, it was pre-ran for 30 minutes at 15 W to ensure that all free ions and unincorporated nucleotides were removed from the gel. The RNA sample was prepared by adding 100  $\mu$ L 0.5X urea dye to 100  $\mu$ L RNA, then boiled for 10 minutes at 100°C and centrifuged. The sample was ran on the gel for 1 hour at 15 W. Upon completion



of the run, the gel was removed and the band corresponding to full-length RNA was identified using ultra-violet (UV) shadowing. The gel was placed on a phosphor screen that fluoresces in the UV range. RNA absorbs UV light so when a UV light source was applied to the gel the screen was shaded where the band appears. This band is then excised from the gel to be further extracted and precipitated.

The RNA was eluted from the excised gel band in order to recover the purified RNA. It was crushed by pushing the gel through a syringe into a 2 mL centrifuge tube. Crush and soak solution (1 mL) was added to the tube, which removes RNA from the gel, followed by 0.5 mL phenol/chloroform, which prevents RNA from degrading. The mixture was incubated for 2 hours at 37°C. Following incubation, the mixture was centrifuged at low speed and the top layer was transferred to a new tube for chloroform extraction and ethanol precipitation. Chloroform (500  $\mu$ L) was added to remove any residual phenol, followed by vortexing for 1 minute and centrifugation for 2 minutes. The top layer containing the RNA was transferred to a new tube and 100  $\mu$ L 3M NaOAc (pH  $\sim$  3.5), 1  $\mu$ L glycogen (20 mg/mL), and 1 mL isopropanol was added. The solution was incubated overnight at -20°C. The RNA was then recovered using the same recovery protocol used earlier (pg. 20), and the RNA concentration was determined using the same nanodrop spectrophotometer as before. The typical RNA yield was approximately 460 ng/ $\mu$ L.

The purity of the recovered RNA was checked on a denaturing PAGE. The components of the gel solution, urea dye, and running buffer are the same as in Table 2 for the previous gel purification step. The prepared gel was pre-ran for 30 minutes at 15 W and the sample was prepared using 5  $\mu$ L recovered RNA and 5  $\mu$ L urea dye. The sample was boiled for 5 minutes at 100°C and centrifuged before loading. The 10  $\mu$ L sample was loaded



and ran for 30 minutes at 15 W. The gel was then transferred into a container with 100 mL 0.5X TBE buffer and 10  $\mu$ L EtBr. The gel was placed on a mutator for 5-10 minutes then photographed with the Biorad Molecular Imager.

#### *RNase Protection Assays*

The probing technique used in this research project is RNase protection assays, specifically using RNase T1, RNase V1, and RNase 1, which all cleave at different sites on the RNA. Samples were made with a) enzyme and RNA and b) enzyme and RNA-tetracycline complex in order to analyze cleavage patterns with and without ligand bound. Before the protection assays were made, all RNA was refolded so that it would be in its native state. To refold the RNA, it was boiled at 100°C for 2 minutes, centrifuged, then placed on ice for ten minutes. All of the components needed for the RNase protection assays can be found in Table 3. The four types of samples made can be found in Table 4, which include samples without ligand bound, samples with ligand bound, the OH ladder, and the T1 ladder.

**Table 3.** *Volumes and concentrations of individual components needed to produce the ribonuclease assays with and without ligand bound, as well as the necessary ladders.*

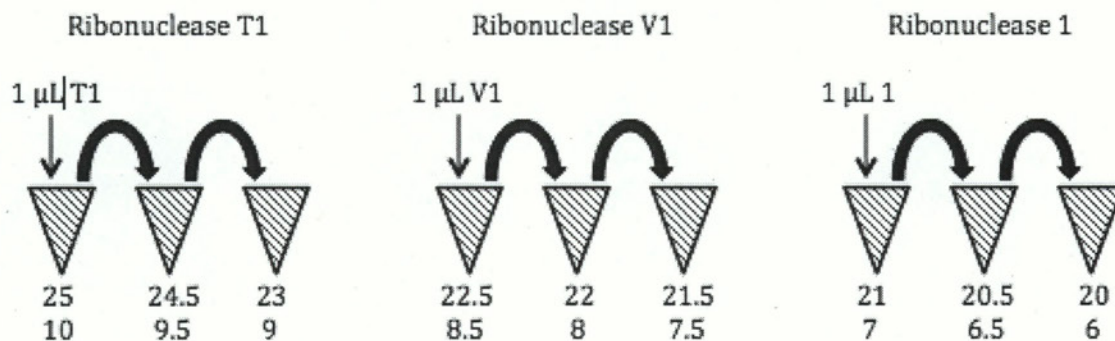
<b>5X Reaction Buffer</b>	<b>10 mM Tetracycline</b>	<b>T1 Buffer</b>	<b>OH Buffer</b>
100 $\mu$ L 1M TrisHCl (pH ~ 8)	50 mg tetracycline	50 $\mu$ L 1M TrisHCl (pH ~ 8)	1 $\mu$ L 1 mM glycine (pH ~ 9.5)
250 $\mu$ L 2M KCl	10 mL DMSO	5 $\mu$ L 0.05% Triton X-100	0.5 $\mu$ L 1M MgCl <sub>2</sub>
5 $\mu$ L 1M MgCl <sub>2</sub>	1 $\mu$ L soln into 1 mL water	Water to 1 mL mark	Water to 1 mL mark
Water to 1 mL mark			

**Table 4.** Volumes and concentrations of each solution that is added in each individual centrifuge tube. A master mix was typically prepared by multiplying each of these volumes by the total number of dilutions that would be made for each type of sample.

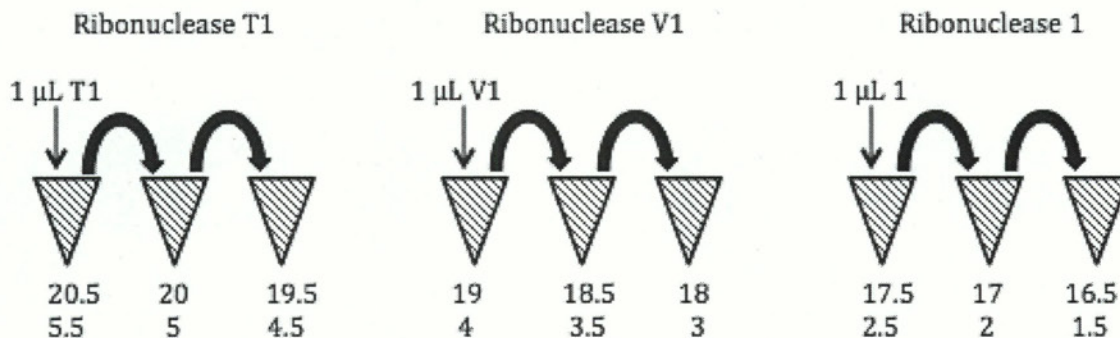
RNA Samples	RNA-Tetracycline Samples	OH Ladder	T1 Ladder
2 $\mu$ L 5X rxn buffer	2 $\mu$ L 5X rxn buffer	1 $\mu$ L RNA	1 $\mu$ L RNA
1 $\mu$ L RNA	1 $\mu$ L RNA	0.5 $\mu$ L tRNA	0.3 $\mu$ L tRNA
5.7 $\mu$ L water	0.3 $\mu$ L tRNA	3.5 1X Alkaline Hydrolysis buffer	3.7 $\mu$ L T1 buffer
0.3 $\mu$ L tRNA	1 $\mu$ L 10 mM tetracycline		
	4.7 $\mu$ L water		

As seen in the schematic in Figure 9, the individual components found in Table 4 were aliquoted into their respective centrifuge tubes. Three serial dilutions for each sample and ladder were done in order to optimize which enzyme concentration produced the clearest cleavage pattern on the sequencing gel. Aside from the OH ladder, the respective ribonuclease (1  $\mu$ L) was added to the first tube in each set of samples, and then a 1:10 dilution was done each time by pipetting 1  $\mu$ L from the first tube into the second tube and then 1  $\mu$ L from the second tube to the third tube. The three enzymes used were RNase T1 (Ambion, 1 U/mL), RNase V1 (Ambion, 0.1 U/mL), and RNase 1 (Ambion, 100 U/mL). Once the enzyme was introduced into the sample, the solution was incubated at room temperature for exactly 15 minutes. As seen in Figure 9, each addition/transfer was done 30 seconds apart (top number) and then each reaction was quenched 30 seconds apart (bottom number) with a 15 minute incubation period in between the start and end of the reaction. To quench each of the ribonuclease samples, 12  $\mu$ L of the quench solution (Table 5) was added to each tube.

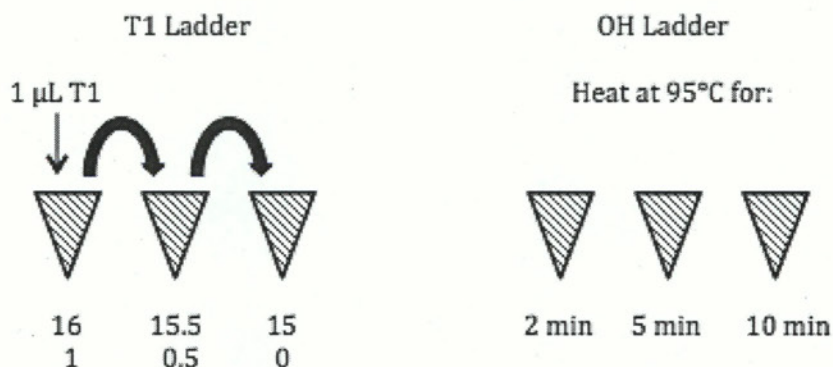
### Samples Without Ligand Bound



### Samples With Ligand Bound



### Ladders



**Figure 10.** Schematic representation of the protocol used for the RNase protection assays. With exception of the OH ladder, the top number under each tube represents the starting time in minutes for that reaction and the bottom number represents the time that the quench solution was added to stop the reaction. The OH ladder is made by heating each solution and adding the quench solution after the allotted amount of time.



To quench the T1 ladder samples, 6.5  $\mu\text{L}$  of the quench solution (Table 5) was added to stop the reaction. The protocol for the OH ladder involved heating each sample at 95°C for a different amount of time to achieve the cleanest fragmentation pattern (Figure 10). The first tube was heated for 2 minutes, the second tube for 5 minutes, and the third tube for 15 minutes. Each reaction was stopped after it's allotted time by adding 6.5  $\mu\text{L}$  of the quench solution (Table 5).

**Table 5.** *Volumes and concentrations of components used in the quench solutions for both ladders and samples.*

<b>Quench Solution for Samples</b>	<b>Quench Solution for Ladders</b>
1 $\mu\text{L}$ 3M NaOAc (pH ~ 3.5)	0.5 $\mu\text{L}$ 3M NaOAc (pH ~ 3.5)
10 $\mu\text{L}$ isopropanol	5 $\mu\text{L}$ isopropanol
1 $\mu\text{L}$ glycogen	1 $\mu\text{L}$ glycogen

Immediately following the quench, every sample was placed in dry ice/EtOH for 30 minutes or incubated overnight at -20°C. Following incubation, each sample was centrifuged for 30 minutes at maximum speed followed by ethanol precipitation as before. Each pellet was dried in the speed vac for 5 minutes, and then used for reverse transcription.

#### *Reverse Transcription*

The reverse transcription protocol consisted of two steps: primer annealing and reverse transcription. The volumes and components of each of the solutions used in the reverse transcription protocol are found in Table 6. To anneal the primers, the RNA pellet of each sample was resuspended in 11  $\mu\text{L}$  water and transferred into a PCR tube. Primer solution (2  $\mu\text{L}$  of 3  $\mu\text{M}$  stock) was added to each tube, which were then placed into the PCR machine and began the 1RTS program. Another PCR tube was added that included only 1  $\mu\text{L}$  clean RNA and 10  $\mu\text{L}$  water. The RTS program follows a 70 minute process:

- Primer annealing for a total of 10 minutes
  - 5 minutes at 65°C
  - 3 minutes at 45°C
  - 2 minutes at 4°C
- At beep, add 6  $\mu$ L RT Master Mix, 1  $\mu$ L RNase Inhibitor (40,000 U/mL), and 1  $\mu$ L EasyScript Plus™ Reverse Transcriptase (200 U/ $\mu$ L)
- Hit enter to begin reverse transcription
  - 1 minute at 45°C
  - 50 minutes at 52°C
  - 10 minutes at 65°C
- Holds the temperature at 4°C for storage

**Table 6.** *Volumes and components needed for reverse transcription protocol.*

<b>3 <math>\mu</math>M Primer</b>	<b>RT Master Mix</b>	<b>Precipitate Solution</b>
3 $\mu$ L 100 $\mu$ M Superstock	400 $\mu$ L RT Buffer	1 $\mu$ L glycogen
Water to 100 $\mu$ L mark	100 $\mu$ L 100 mM DTT	2 $\mu$ L 3M NaOAc (pH ~ 3.5)
	100 $\mu$ L 10 mM dNTP mix	50 $\mu$ L 95% EtOH

Upon completion of reverse transcription, the solution in each PCR tube was transferred to a 1.5 mL centrifuge tube and 53  $\mu$ L of precipitate solution was added to quench the reaction. The tubes were placed on dry ice/EtOH for 30 minutes to effectively end the reaction. The samples are then recovered using the same recovery procedure as before (pg. 20), but instead of resuspending the samples in 100  $\mu$ L water, the samples were stored at -20°C until the sequencing gel was prepared to be ran.

#### *RNA Sequencing Gel*

Sequencing gels were used to visualize the cleavage pattern of the RNA following ribonuclease digestion with and without ligand bound. Thin (41 cm), polyacrylamide gels were used with 0.35 mm spacers and analyzed using the LI-COR 4300 DNA Analyzer. The volumes and components of the gel solution, TBE buffer, and urea dye used can be seen in Table 7.

**Table 7.** Volumes and components of the gel solution, TBE buffer, and urea dye used for the thin, polyacrylamide sequencing gel.

<b>0.8X TBE Buffer</b>	<b>0.8X Urea Dye</b>	<b>Gel Solution</b>
80 mL 10X TBE	0.36 g urea	14.4 g urea
Water to 1 L mark	80 $\mu$ L 10X TBE	10 mL 40% acrylamide/bisacrylamide
	25 $\mu$ L bromophenol blue	3.2 mL 10X TBE
	Water to 1 mL mark	Water to 40 mL mark
		320 $\mu$ L 10% ammonium persulfate
		32 $\mu$ L TEMED

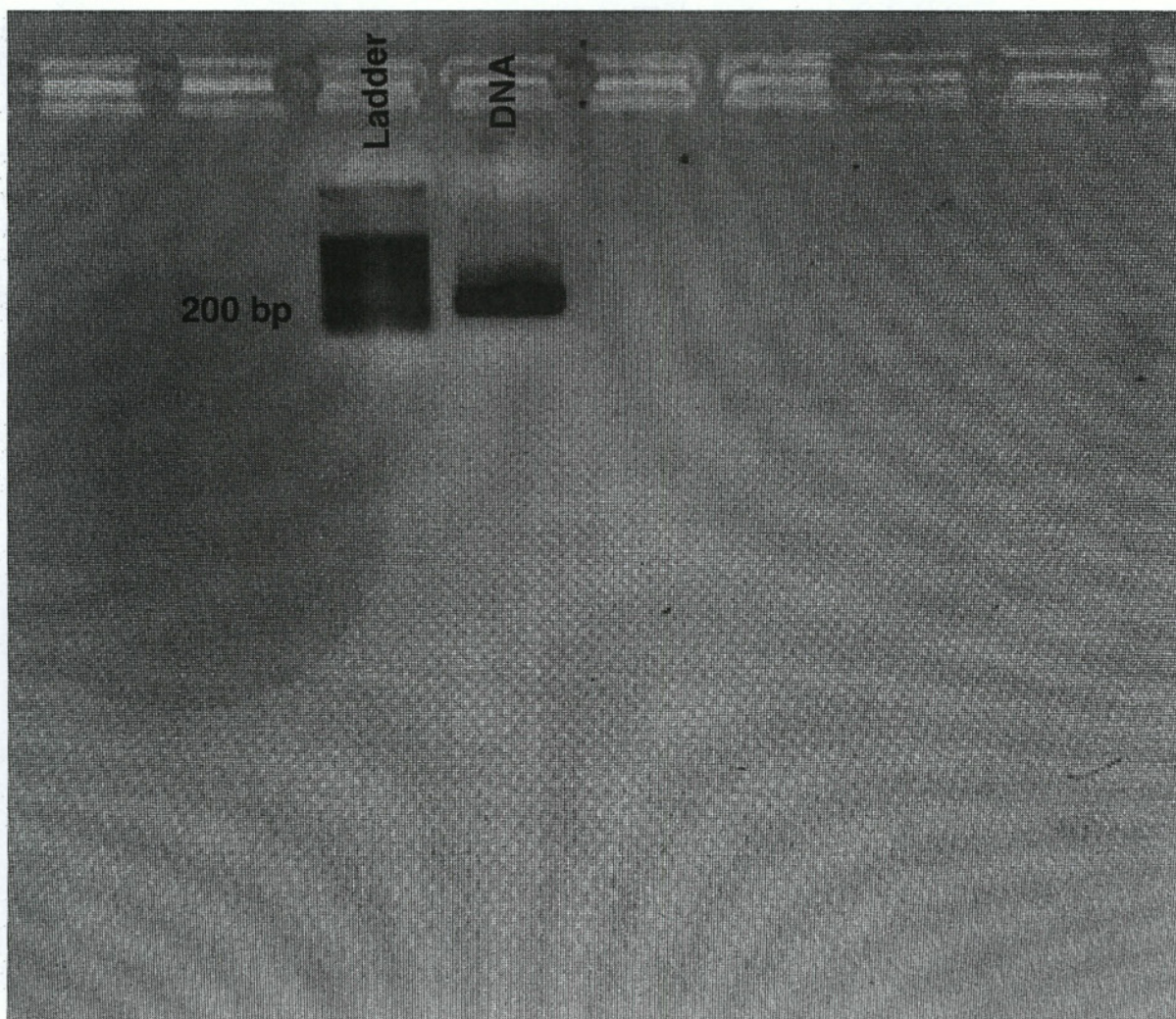
Once the gel polymerized (~ 45 minutes), the gel was placed in the DNA Analyzer along with 0.8x TBE buffer. The gel was pre-ran for 20 minutes to ensure that any contamination was eluted from the gel. The recovered samples were resuspended in 5  $\mu$ L 0.8X urea dye, boiled at 100°C for 5 minutes and centrifuged. The appropriate volume of each sample and ladder was loaded into the wells so that there were no spaces in between. The gel was then run for 4 hours.

## Results

### *ykkCD Riboswitch RNA Synthesis*

After amplifying the ykkCD DNA template using PCR technology and purifying the product per suggestion by the manufacturer, the yield was found to be 194.2 ng/ $\mu$ L per 1 mL of PCR product. The quality of the DNA product was checked using agarose gel electrophoresis and a strong band was found around 200 bp, which matches the size of the DNA template (Figure 11). The left lane on the gel shows the ladder that was used as a standard, and the right lane shows the purified DNA product. The solitary band is indicative of a relatively clean product since there are no other bands that suggest any impurities are present.





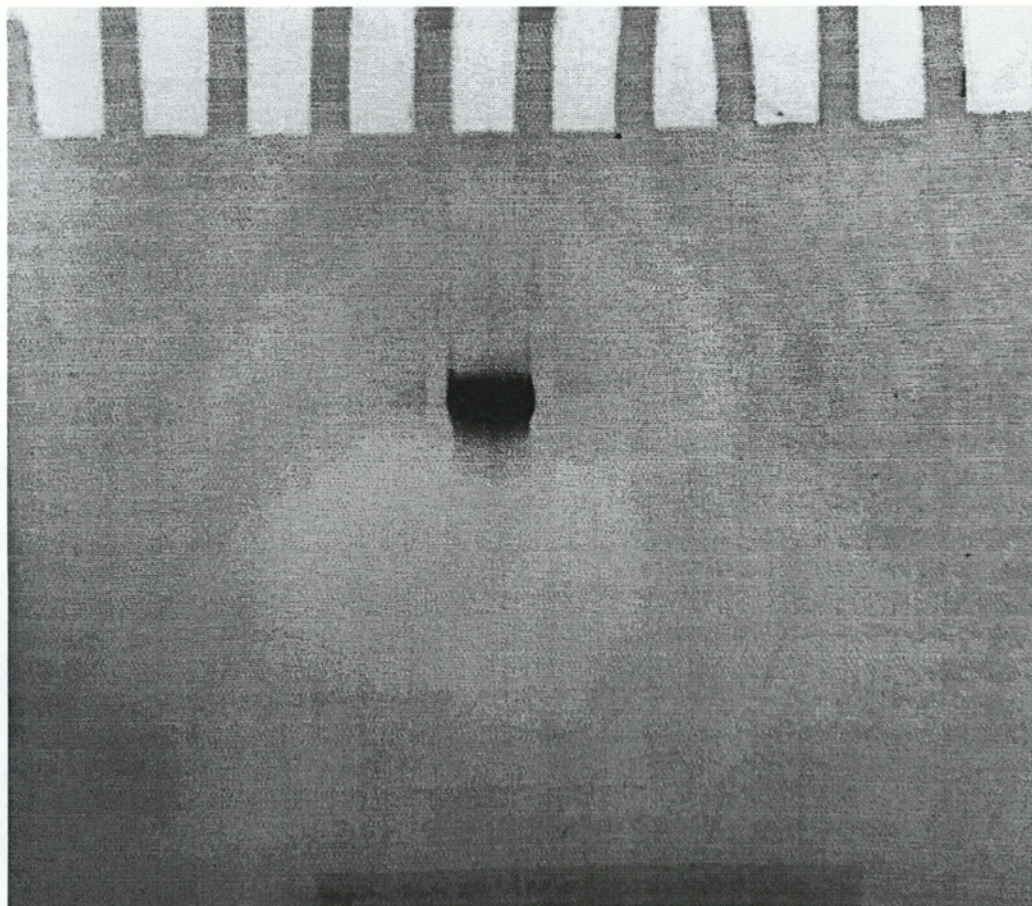
**Figure 11.** Image collected using the Biorad Molecular Imager to analyze the purity of the DNA product compared to a standardized ladder on an agarose gel.

The RNA product was then transcribed using this DNA template and the precipitated RNA was purified using gel purification. The yield of the purified RNA was found to be 459.3 ng/ $\mu$ L per 0.5 mL of the transcription sample. To find the molar concentration, the absorbance at 260 nm was divided by the molar extinction coefficient of ykkCD RNA, which is 1149800 (Equation 1). The concentration of the RNA was found to be  $7.99 \times 10^{-6}$  M.

$$\text{Equation 1: } \frac{\text{Absorbance @ 260 nm}}{\text{Molar extinction Coefficient}} = \frac{9.187}{1149800} = 7.99 \times 10^{-6} \text{ M RNA}$$



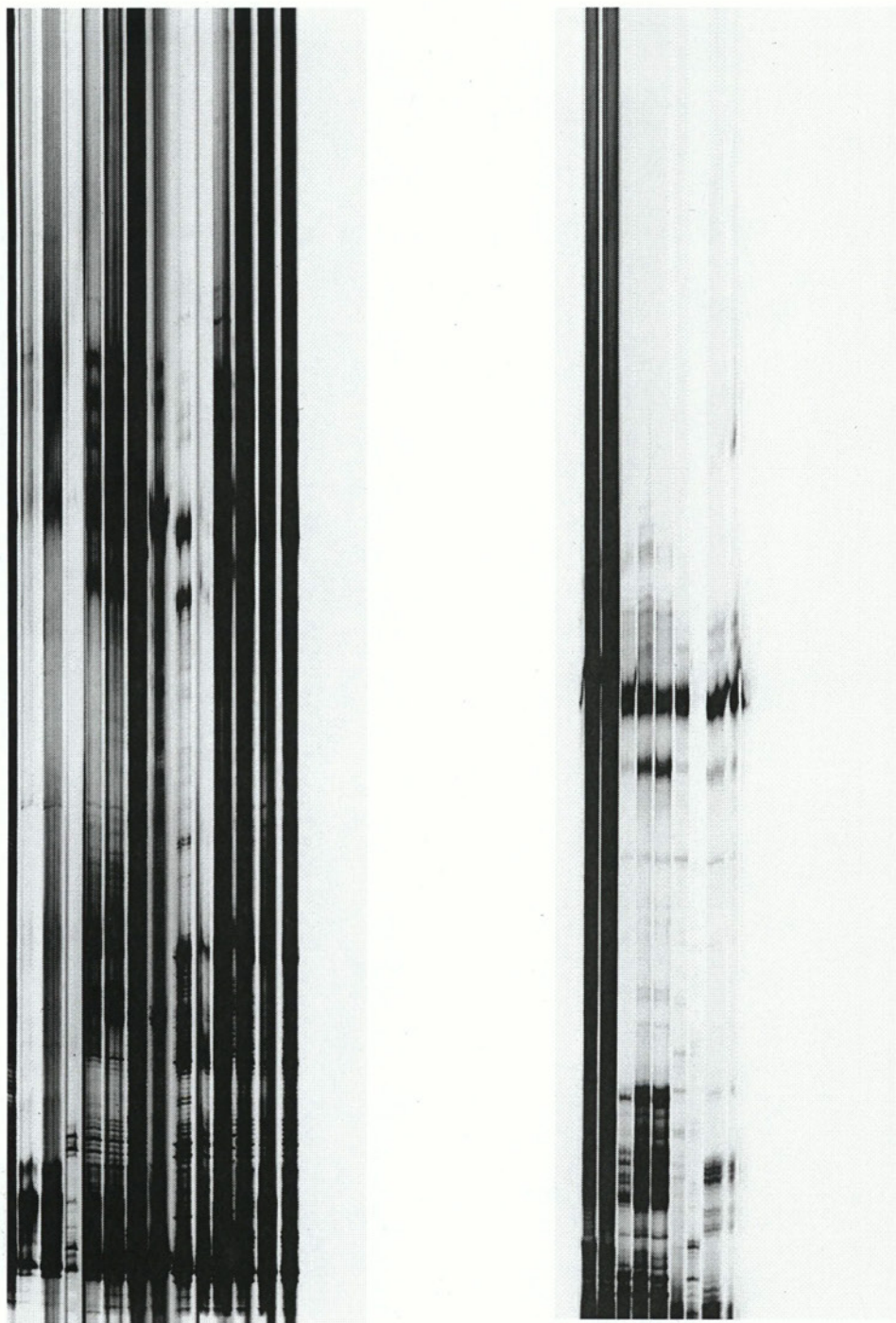
The purity of the RNA product was checked on a denaturing PAGE. The presence of one solid band suggests that the product was pure (Figure 12).



**Figure 12.** Image collected using the Biorad Molecular Imager to check the purity of the RNA product on a denaturing PAGE gel.

#### *Optimization of RNA Sequencing Gels*

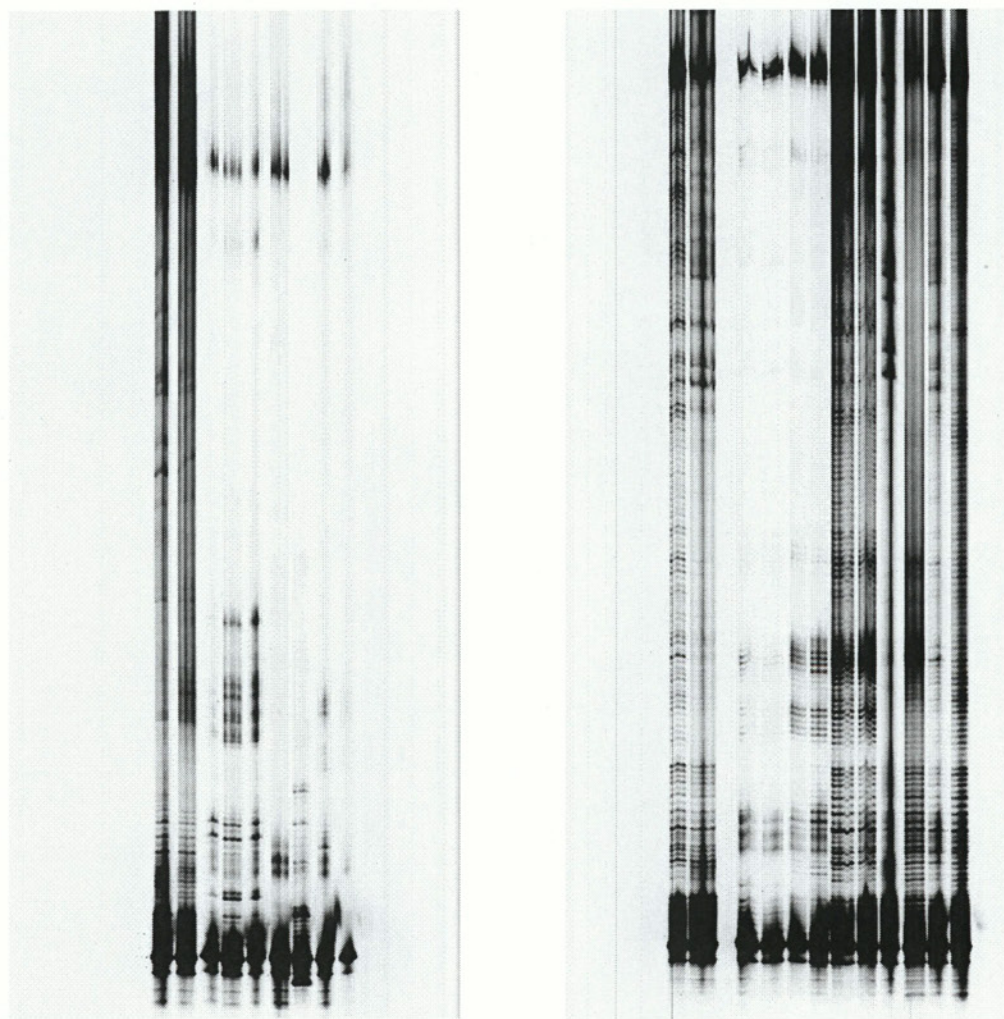
The optimization of the sequencing gels began with the determination of the appropriate amount of sample to load in order to achieve the clearest cleavage pattern. When the gel is overloaded with sample, it is difficult to read and analyze the distinct bands because the lanes show up as dark streaks (Figure 13). Occasionally, white lines would



**Figure 13.** Representative gels that exhibit overloading in multiple lanes. In the first gel (left), the majority of the lanes are overloaded. In the second gel (right), the first two lanes are overloaded.



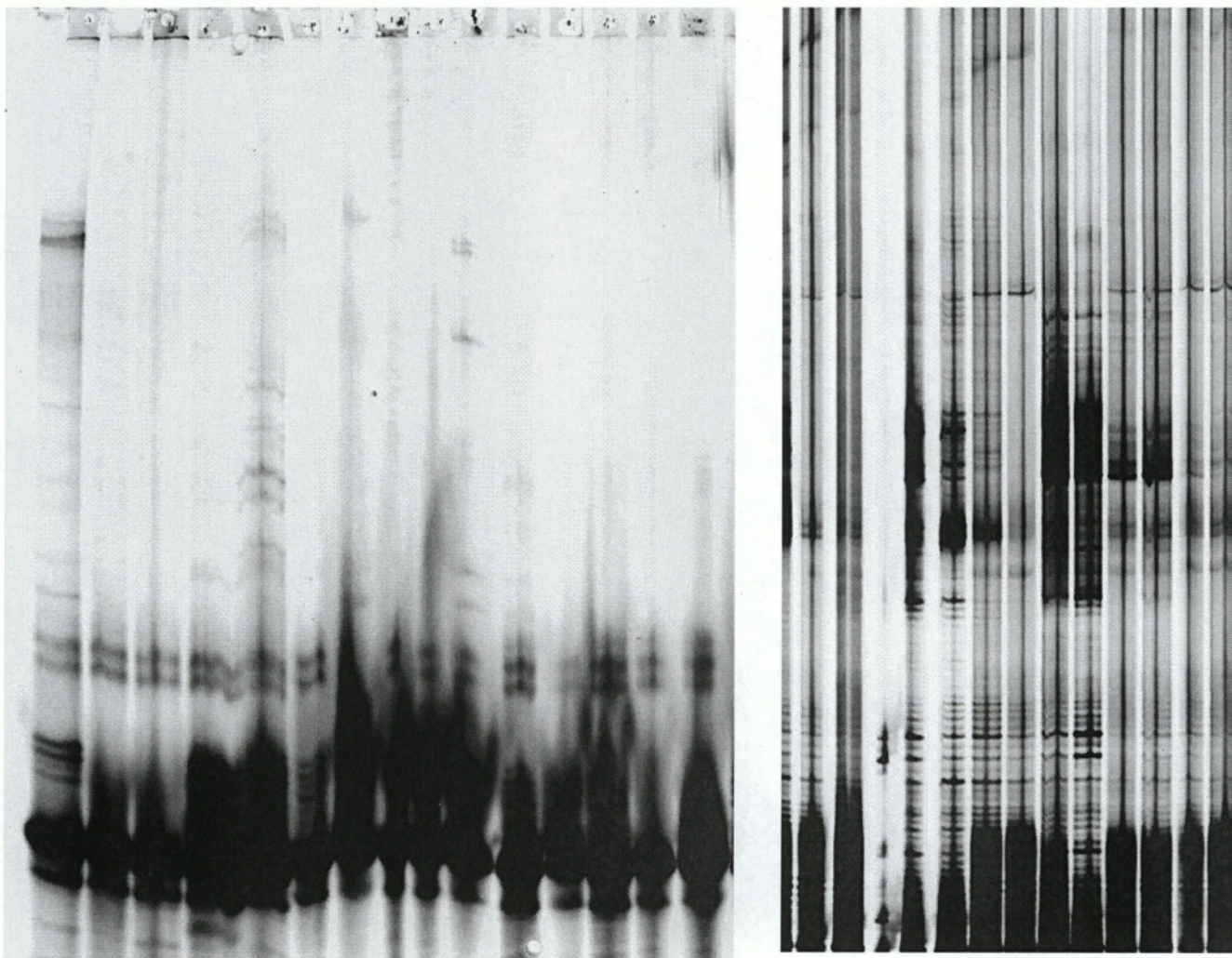
appear within the solid black streaks, which is another indication of overloading the gel. As seen in the gels in Figure 13, when samples are overloaded their cleavage patterns disappear and no information can be obtained. In both of these gels, 8 picomoles of the sample were loaded in the overloaded lanes. Since this appeared to be too great of an amount to load, several gels were ran using different amounts of samples in an attempt to optimize the appropriate load. It was found that the optimal amount of sample to load fell within the range of 0.6 - 2 picomoles (Figure 14).



**Figure 14.** The first gel (left) was loaded with 2 picomoles of sample while the second gel (right) was loaded with 0.6 picomoles of sample.



A few of the preliminary gels showed that the sample was being overdigested by the enzyme, which made it difficult to read any cleavage patterns. Therefore, approximately 0.3  $\mu$ L of tRNA was added to each sample to protect it from over digestion. The nucleases cleave both ykkCD RNA and tRNA thus ykkCD RNA will not be cleaved as much. A few representative gels (Figure 15) show the difference in the cleavage patterns before and after the addition of tRNA.



**Figure 15.** The gel on the left was run before tRNA was introduced into the sample before reverse transcription occurred. The gel on the right was run with samples that had tRNA added to the master mix prior to reverse transcription. The cleavage pattern is much more distinct, which indicates that the sample was not overdigested.

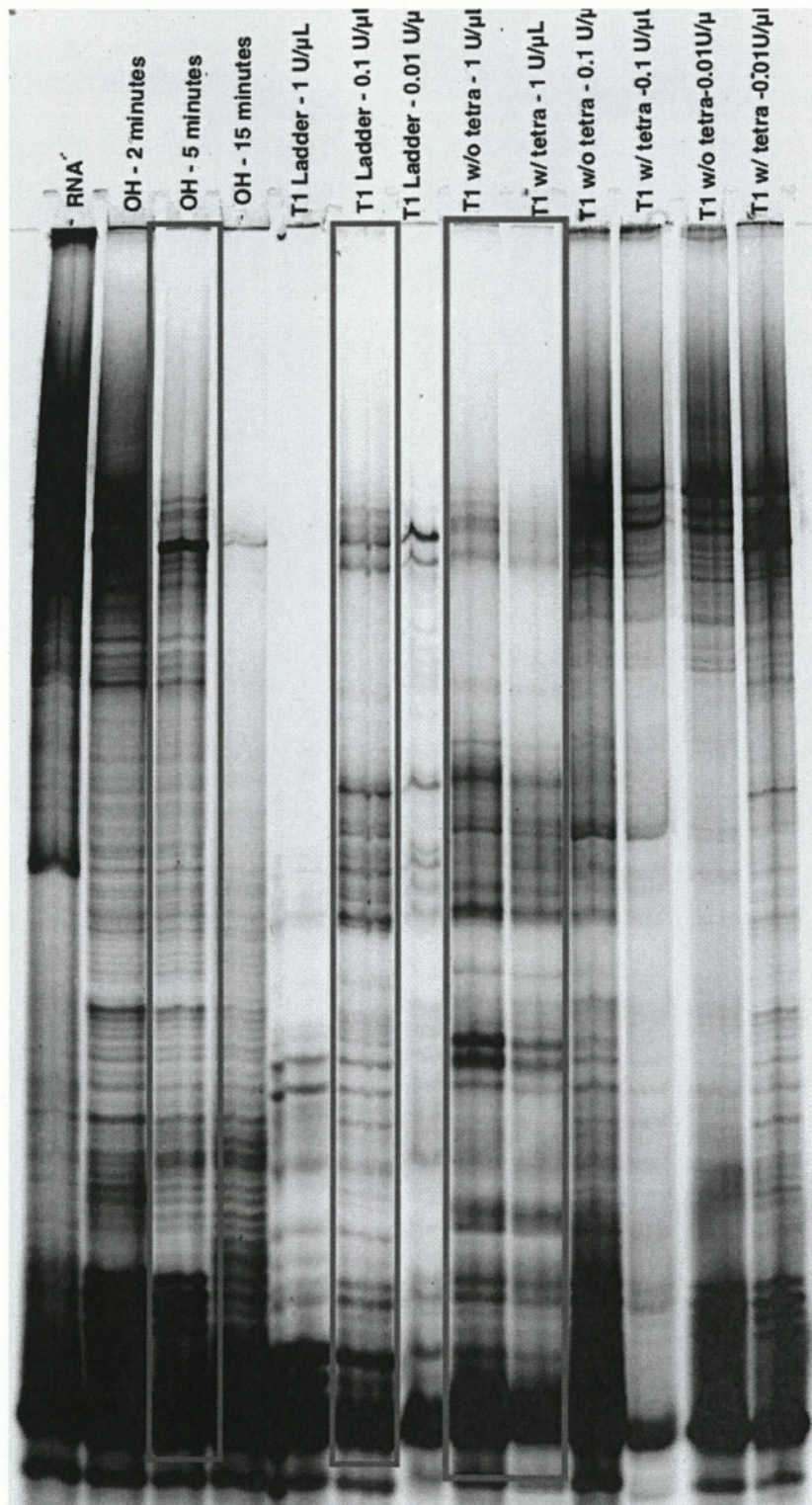
The final step of optimization was to determine the enzyme concentration that generated the clearest cleavage pattern on the sequencing gel. As shown in Figure 10 in the Materials and Methods section, a 1:10 serial dilution was done for each of the enzymes and the T1 ladder so that it could be determined which enzyme concentration cleaved the sample optimally. The same was done for the OH ladder, but instead of a serial dilution, the samples were heated at 95°C for 2, 5, and 15 minutes. The enzyme concentrations were optimized (Table 8) by visually inspecting cleavage patterns on gels.

**Table 8.** *Optimized dilutions and concentrations of each sample.*

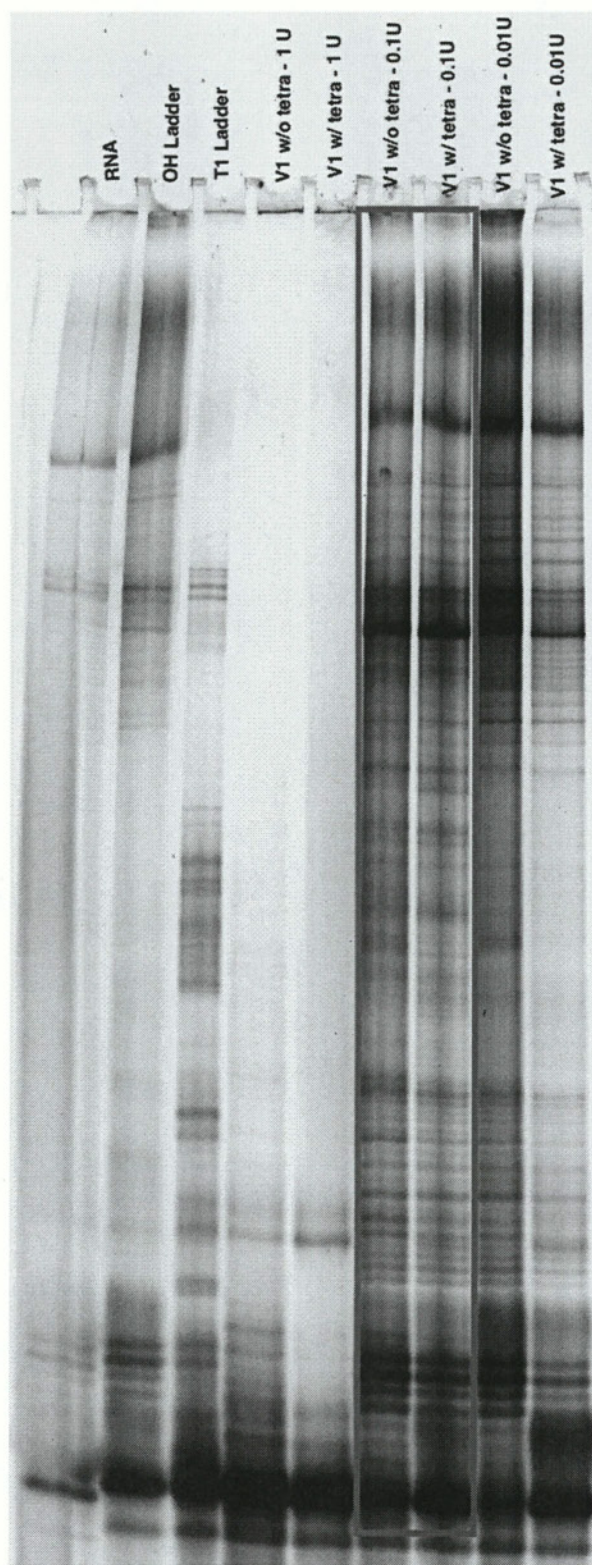
<b>Sample</b>	<b>Dilution</b>	<b>Concentration</b>
OH Ladder	-	Heated for 5 minutes
T1 Ladder	2	0.1 U/ $\mu$ L
RNase T1	1	1.0 U/ $\mu$ L
RNase V1	2	0.01 U/ $\mu$ L
RNase 1	3	1.0 U/ $\mu$ L

The representative gels for each of the optimizations can be seen in Figures 16-18. In these figures, the optimized samples are outlined by a border. The OH ladder generated the clearest cleavage pattern when it was heated for 5 minutes (Figure 16). Incubation for 5 minutes yielded more distinct cleavage patterns than the 2 or 15 minute incubations. Similarly, the T1 ladder produced the most distinct bands when the enzyme concentration was 0.1 U/ $\mu$ L. The RNase T1 was optimized at 1.0 U/ $\mu$ L (Figure 16), RNase V1 was optimized at 0.01 U/ $\mu$ L (Figure 17), and the RNase 1 was optimized at 1.0 U/ $\mu$ L (Figure 18).



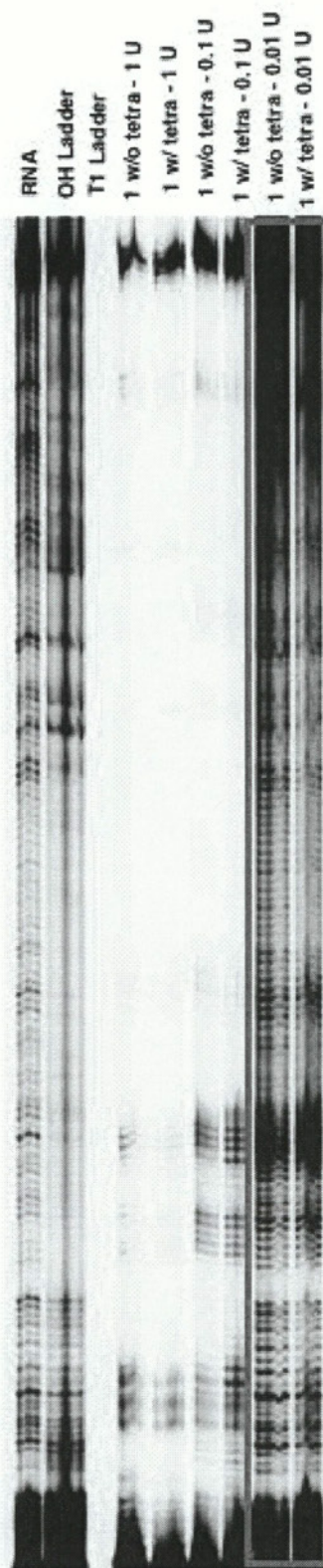


**Figure 16.** Optimized samples of the OH ladder, T1 ladder, and RNase T1 are outlined in red. These dilutions produced the clearest cleavage patterns and were used for all further research.



**Figure 17.** Optimized samples of RNase V1 without and with tetracycline. This dilution was used for all further research.





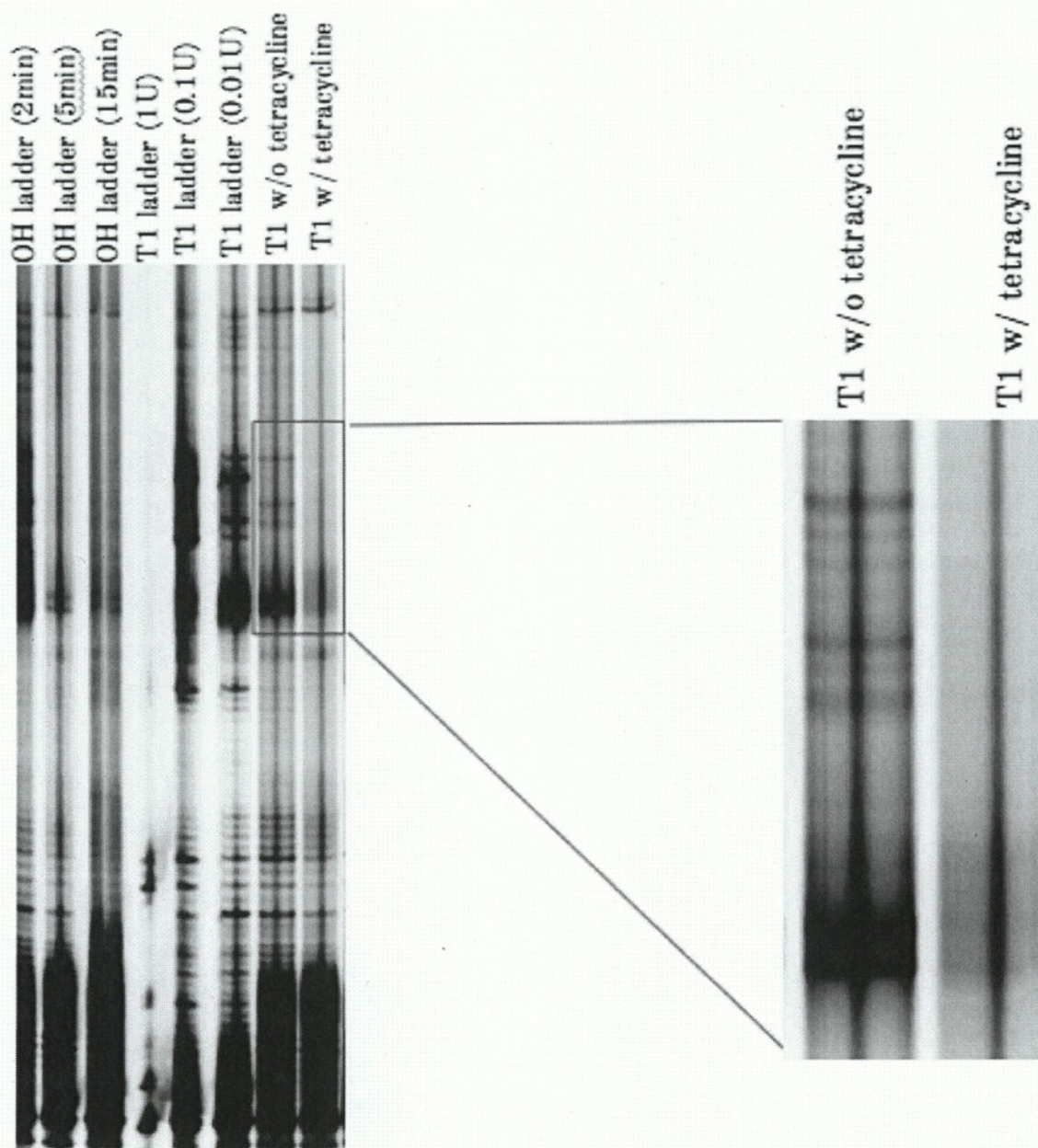
**Figure 18.** Optimized samples of RNase 1 without and with tetracycline. This dilution was used for all further research.



### *Detection of Structural Change*

Once the enzyme concentrations and load amounts were optimized, sequencing gels were run to detect any structural changes that occurred due to the binding of tetracycline. As mentioned earlier, RNase T1 cleaves after every G nucleotide in single-stranded RNA, RNase V1 cleaves double-stranded RNA, and RNase 1 cleaves single-stranded RNA. Ideally, one would resolve a gel with a clean cleavage pattern for each ladder and each RNase with and without tetracycline in order to determine the structural change caused by tetracycline binding. Because RNase T1 cleaves after every G nucleotide, each band that appears in the T1 ladder corresponds to a G nucleotide. Therefore, a clean T1 ladder can be used to match the cleavage pattern caused by a nuclease to the nucleotide sequence of ykkCD.

To assess structural change, the digested samples are run on the same gel to look for areas of protection or enhancement within the cleavage patterns. When protection occurs, the sample without tetracycline will be cleaved more heavily than the sample that is bound by tetracycline, which will produce darker bands in the cleavage pattern. The lighter band in the sample with tetracycline suggests that the binding of tetracycline protected those specific nucleotides from being cleaved by the enzyme. When enhancement is detected, the opposite has occurred. The lane that contains a sample without tetracycline will have a band that is lighter than the corresponding band in the lane that contains the sample with tetracycline. The darker band in the sample with tetracycline indicates enhanced cleavage, which suggests that the binding of tetracycline caused a structural change that increased the vulnerability of those nucleotides to cleavage. Sequencing gels were produced that contained evidence of protection (Figures 19 & 20) as well as enhancement (Figures 21 & 22).

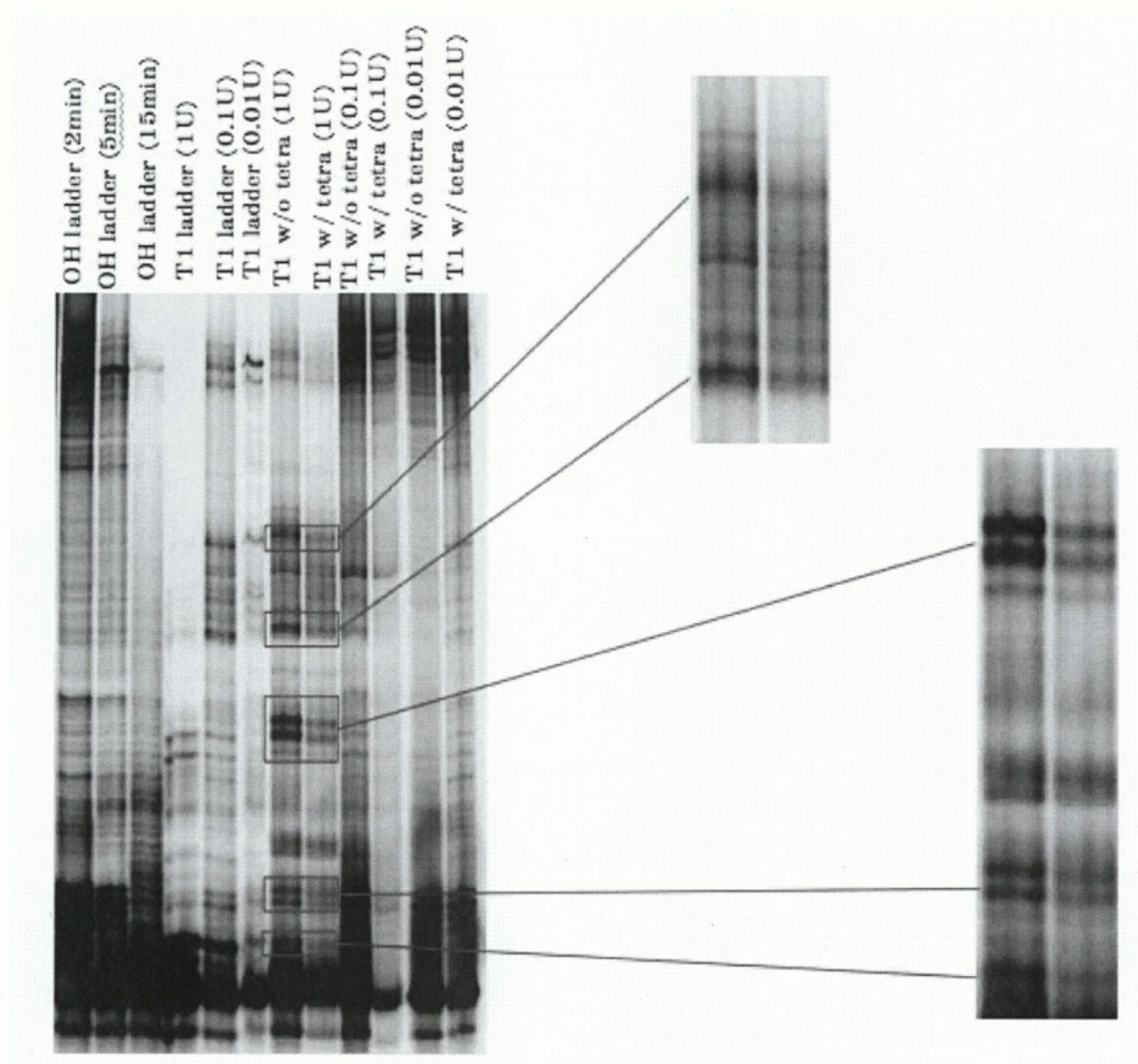


**Figure 19.** Evidence of protection in RNase T1 samples without and with tetracycline.

In Figure 19 there appears to be protection occurring at several G nucleotides. Both of the samples appear to have been loaded equally so any change in the intensity of the bands can be contributed to protection or enhancement. It is evident in the sample with tetracycline that there is significantly less cleavage occurring, which suggests that either tetracycline binds at or near those nucleotides or it binds in such a way that a structural



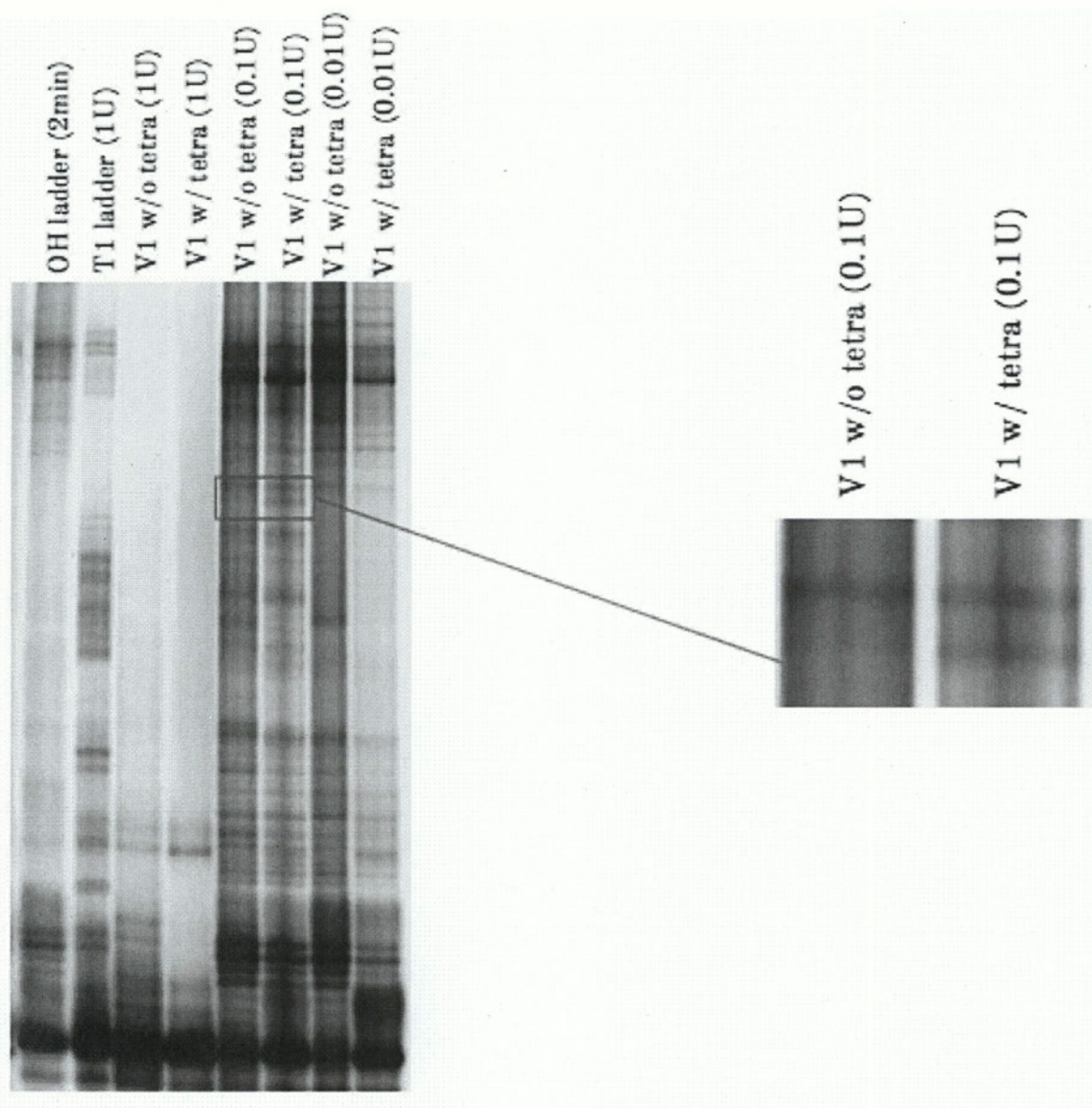
change occurs that protects those specific sites. Other parts of the RNA do not appear to be affected by tetracycline binding. The T1 ladder is slightly overloaded so it could not be used to compare to the RNA sequence to determine which specific G nucleotides in the sequence are being protected.



**Figure 20.** Another example of a gel in which there is evidence of protection in RNase T1 samples without and with tetracycline.

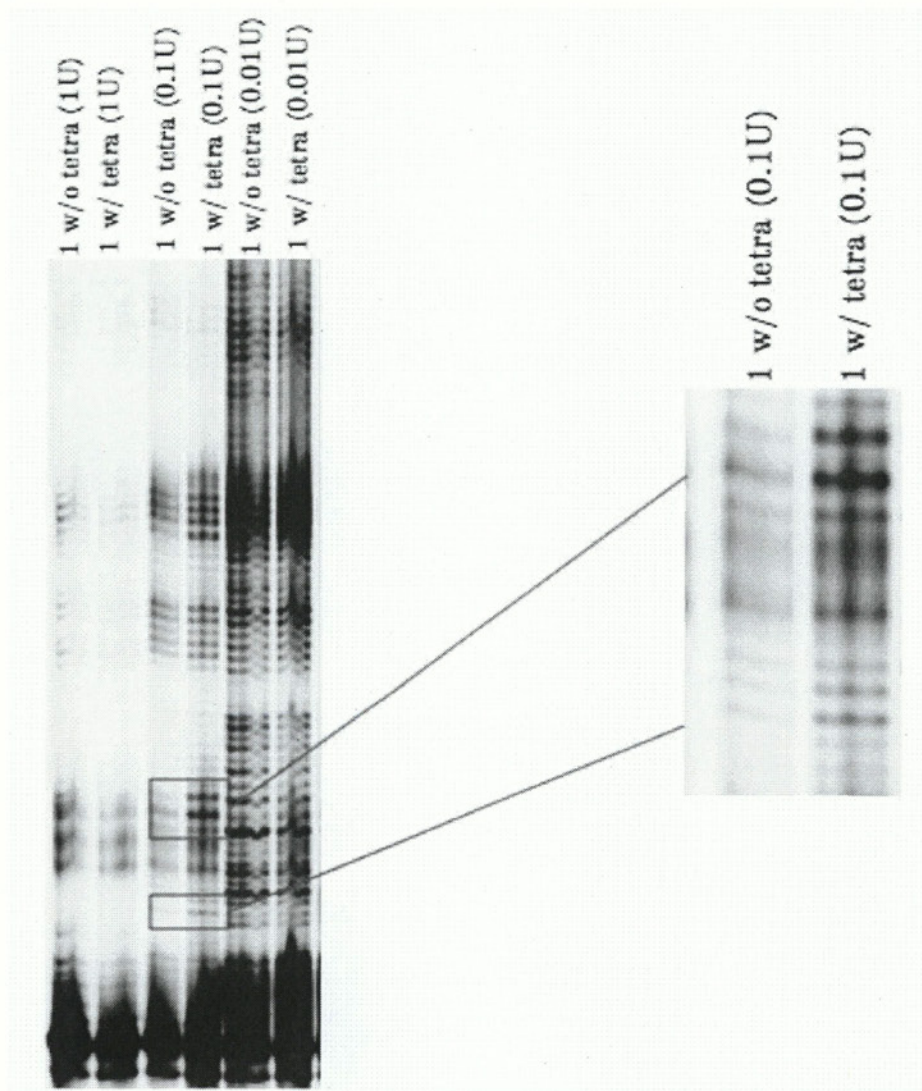


Similar to the previous gel, the T1 samples without and with tetracycline in Figure 20 exhibit protection in several areas of the sequence. The intensity decreases when tetracycline is added, which is once again indicative of a structural change caused by binding of tetracycline. The T1 ladder in this gel produced a more definite cleavage pattern; however, there was a slight amount of waving within the gel that makes interpretation difficult.



**Figure 21.** Evidence of enhancement in RNase V1 samples without and with tetracycline.

In Figure 21, there is evidence of a slight enhancement within the V1 samples. As seen in the enlarged segment, a band appears in the sample with tetracycline that was not present in the sample without tetracycline. This indicates that a structural change occurred that made those specific nucleotides vulnerable to cleavage by RNase V1. This enzyme specifically cleaves double-stranded RNA. Therefore, this segment of enhancement suggests that at that specific point in the RNA sequence the binding of tetracycline caused those nucleotides to base pair and become double-stranded instead of single-stranded.



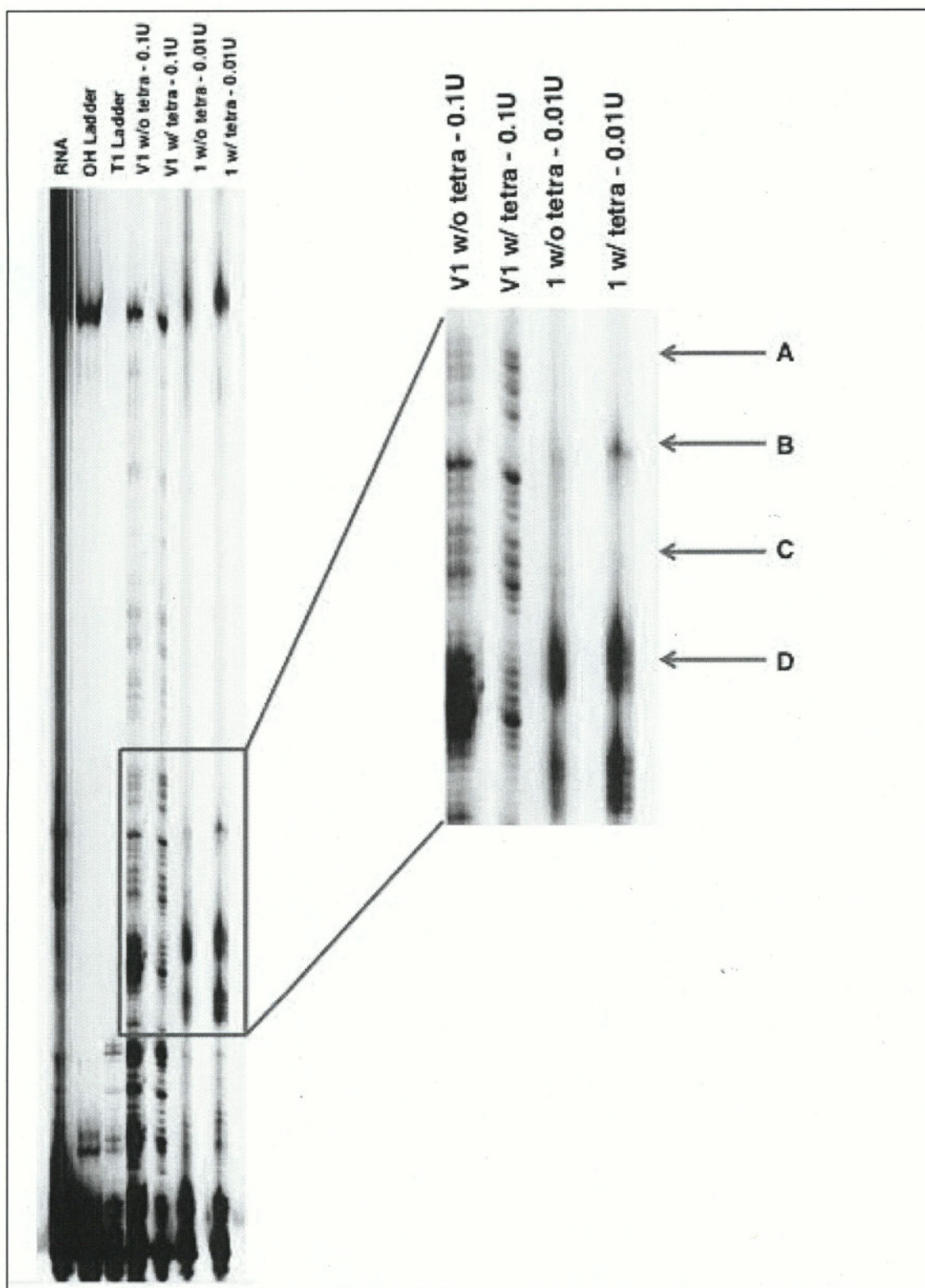
**Figure 22.** Evidence of enhancement in RNase 1 samples without and with tetracycline.



RNase 1 specifically cleaves single-stranded RNA. In Figure 22, there are several examples of enhancement that occur when tetracycline is added to the sample. This indicates that the binding of tetracycline causes a structural change that separates double-stranded RNA into single-stranded RNA; thus allowing the single-stranded RNA to be cleaved by the enzyme. Unfortunately the ladders that were used on this gel did not produce promising cleavage patterns so that exact nucleotides that were being altered could not be identified. However, between the gels seen in Figures 21 and 22 it is clear that there are structural changes occurring that transform areas of single-stranded RNA into double-stranded and double-stranded RNA into single-stranded RNA.

Because of the ability of both RNase V1 and RNase 1 to cleave specific areas of a sequence, it is highly beneficial to produce a sequencing gel that contains clear cleavage patterns for both samples, with and without tetracycline. By comparing cleavage patterns produced by these enzymes areas of structural change can be detected because they have complementary cleavage patterns. Areas of the RNA that are double-stranded before tetracycline is added will show up as distinct bands in the RNase V1 samples but not in the RNase 1 samples. In a complementary manner, any areas of single-stranded RNA will not show up as bands in the RNase V1 samples, but will appear in the RNase 1 samples. When tetracycline is added to the samples, if a conformational change occurs that alters single- and double-stranded RNA in those areas then the bands that once showed up in the RNase V1 sample will now disappear because those nucleotides became single-stranded, thus protected from RNase V1. In a corresponding manner, bands will now appear at those same locations in the RNase 1 sample that were not present before because the RNA is now single-stranded and capable of being cleaved by RNase 1.





**Figure 23.** Comparison of RNase V1 and RNase 1 samples without and with tetracycline. The results suggests that tetracycline binding leads to a structural change.

As seen in Figure 23, a sequencing gel was produced that contained complementary cleavage patterns between RNase V1 and RNase 1. The portion of the gel indicated by arrow A shows cleavage of the RNA by RNase V1 which means that those portions of the RNA sequence are double-stranded. The lack of bands in the RNase 1 sample supports that statement since RNase 1 cannot cleave double-stranded RNA. There is also a slight enhancement in the RNase V1 sample with tetracycline, which suggests that RNA becomes more structured.

The portion of the gel indicated by arrow B shows evidence of enhancement in the RNase 1 sample when tetracycline is added. The lack of bands in the RNase V1 samples suggests that the RNA at that location becomes single-stranded upon tetracycline binding. The pattern then changes at arrow C and distinct bands appear in the RNase V1 samples. There also appears to be a slight enhancement within those bands when tetracycline is added. The pattern in the RNase 1 samples react complementarily. The faint bands at arrow C in the RNase 1 sample without tetracycline disappear when tetracycline binds. This suggests that when tetracycline binds to the RNA it is protected from RNase 1 but more susceptible to RNase V1, mostly likely because a structural change is occurring that transforms any single-stranded RNA in that area to double-stranded RNA.

Lastly, the portion of the gel that is indicated by arrow D shows both protection in the RNase V1 samples and corresponding enhancement in the RNase 1 samples. Although the changes in intensity of the bands are slight, the complementary changes in both samples when tetracycline is added suggest that another structural change is occurring that is separating the double-stranded RNA into single-stranded RNA. Therefore the RNA is more susceptible to cleavage by RNase 1 than it is by RNase V1.



## Discussion

Throughout the course of this research project, ykkCD RNA was subjected to ribonuclease protection assays followed by reverse transcription to resolve the corresponding cleavage patterns on a sequencing gel. By observing the changes in the cleavage patterns in the presence and absence of tetracycline the goal was to determine how the binding of tetracycline altered the secondary structure of the ykkCD RNA. Areas of protection and enhancement caused by tetracycline binding can then be compared to the nucleotide sequence of ykkCD when nuclease-treated samples and the corresponding ladders are resolved clearly on the same gel.

Ribonuclease protection assays are commonly used and their protocols are well established. They are beneficial to use because each RNase has cleaving specificity. Because RNase T1 cleaves after every G nucleotide, it helps the researcher orient the cleavage pattern within the RNA sequence. More importantly, RNase V1 and RNase 1 work in a complementary manner and are sensitive to changes in secondary structure. Because RNase V1 cleaves double-stranded RNA and RNase 1 cleaves single-stranded RNA the cleavage patterns should change in a synchronized manner if a structural change occurs. When these two enzymes are used in conjunction they can offer a lot of information about structural changes that are occurring due to metabolite binding.

During this research project, the assay protocols were optimized for RNase T1, V1, and 1 along with the protocols for both ladders. Several sequencing gels were ran with all three of the dilutions of each ladder and sample (1U, 0.1U, 0.01U) to determine which concentration of each species produced the clearest cleavage patten. The optimized enzyme concentrations are summarized in Table 9.

**Table 9.** *Optimized enzyme concentrations for each of the nucleases and the optimized incubation time for the hydroxyl ladder.*

Sample	Concentration
OH Ladder	Heated for 5 minutes
T1 Ladder	0.1 U/ $\mu$ L
RNase T1	1.0 U/ $\mu$ L
RNase V1	0.01 U/ $\mu$ L
RNase 1	1.0 U/ $\mu$ L

Refer to Figures 16, 17, and 18 for sequencing gels that used the optimized protocols. Once each sample was optimized, the focus shifted to producing gels with clean cleavage patterns for each sample to begin comparing the patterns in the presence and absence of tetracycline. Unfortunately, due to time constraints in the lab it was difficult to produce a large volume of gels that contained distinct, readable patterns for every sample. In many cases, the RNase samples with and without tetracycline would generate clean cleavage patterns but the OH and T1 ladders would not be as clear, or the opposite would occur. Until both of the ladders can be consistently read on a gel with enough clarity to identify each nucleotide, it is not possible to interpret which specific nucleotides in the ykkCD RNA sequence are being bound or structurally altered.

The data collected showed promising evidence of protection and enhancement when tetracycline was introduced into the samples. Refer to Figures 19-23 for representative gels that exhibit these changes. We were unable to determine which specific nucleotides were being protected or enhanced due to the lack of corresponding ladders on the same gel, but it is clear that tetracycline binding causes changes to the secondary structure in specific areas within the sequence.



Although each of the enzymes have adequate resolution of their fragmentation patterns, RNase V1 and RNase 1 used simultaneously seem to show the most promise and the clearest evidence of structural changes since they cleave in a complementary manner. Therefore, I think it will be most useful in the future to pursue the study of these two ribonucleases in tandem in order to determine how the secondary structure is changing. It also may be useful in future research to try other methods of visualizing the fragmentation patterns, such as radiolabeling with  $^{32}\text{P}$  or trying other fluorescent end-labeling techniques, to see if they produce clearer cleavage patterns. However, using the optimized ribonuclease protocols determined in this research project, I think it will be possible to identify which specific nucleotides in the ykkCD RNA sequence are being affected by tetracycline binding using these ribonuclease protection assays. Moreover, I think that it is possible to determine the tetracycline binding site on ykkCD RNA in future research using the data collected and methods optimized throughout this research project.

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